MECHANISMS OF NMDA RECEPTOR MODULATION BY METABOTROPIC RECEPTORS IN THE HIPPOCAMPUS

DISSERTATION

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"A journey of a thousand li begins with a single step."

Lao Tsi

Dedicated to my parents

Zusammenfassung

Glutamat, der wichtigste erregende Neurotransmitter im Gehirn, bindet sich an eine Vielfalt von neuronalen Rezeptoren. Einer dieser Rezeptoren, der N-Methyl-D-Aspartat-Rezeptor (NMDAR), ist von speziellem Interesse wegen seiner zentralen Rolle in der Entwicklung des Nervensystems, bei der synaptischen Plastizität sowie bei Lernprozessen. Ferner können Probleme bei der NMDAR-vermittelten Signalübertragung zu neurologischen Ausfällen wie Schizophrenie, Epilepsie, Parkinsonsche Krankheit und ischämischer Hirnschlag führen. Der NMDAR gehört zu einer Familie von ionotropen Glutamatrezeptoren, zu denen auch die AMPA-Rezeptoren (α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionsäure) und die Kainatrezeptoren gehören, die alle drei an der Übermittlung von raschen synaptischen Antworten beteiligt sind.

Die NMDAR sind postsynaptisch lokalisiert, wo sie zu grossen Proteinkomplexen angeordnet sind (zusammen mit anderen Rezeptoren und Enzymen), die die Transduktionsmachinerie - im Elektronenmikroskop als postsynaptische Dichte (PSD) erkennbar - bilden. Obwohl die NMDAR weniger zahlreich sind als die AMPA Rezeptoren und nur einen Bruchteil des synaptischen Stromes leiten, nehmen sie eine entscheidende Funktion bei der Regulierung der synaptischen Übertragung wahr. Sehr viele Moleküle und Transduktionskaskaden regulieren die Funktion der NMDAR, um deren Aktivität innerhalb enger physiologischer Grenzen zu halten (oder: in einem engen physiologischen Bereich zu halten). G-Protein-gekoppelte Rezeptoren, auch metabotrope Rezeptoren genannt, sind via die Aktivierung spezifischer intrazellulärer Prozesse sehr wichtig für die Kontrolle der NMDAR Steuerung. Die primären Mechanismen der Modulierung der NMDAR Funktion, mit spezieller Berücksichtigung der durch die Stimulation der metabotropen Rezeptoren induzierten Signaltransduktionskaskaden werden im ersten Kapitel zusammengefasst.

Im zweiten Kapitel präsentiere ich experimentelle Daten, die zeigen, dass die Richtung und das Ausmass der NMDAR-Modulation durch G_q-gekoppelte

metabotrope Rezeptoren in kritischer Abhängigkeit von der intrazellulären Kalziumpufferung stehen. Ferner konnte beobachtet werden, dass diese Modulation zellspezifisch ist. Die Stimulation metabotroper Rezeptoren führt in CA1 Pyramidenzellen zu einer Potenzierung der NMDAR, während eine Hemmung dieser Rezeptoren in den CA3 Pyramidenzellen resultiert. Diese Erkenntnisse könnten einen Einblick in die Mechanismen ermöglichen, welche für die viel stärkere Empfindlichkeit von CA1 gegenüber CA3 Pyramidenzellen bei der zerebralen Ischämie verantwortlich sind.

Im dritten Kapitel wird ein negativer modulatorischer Signalweg beschrieben, der die Hemmung von NMDAR-Antworten durch Muskarin in CA3 Pyramidenzellen vermittelt. Bisher war lediglich eine Potenzierung der NMDAR-Antworten via die metabotrope Aktivierung von Tyrosinkinasen bekannt. Die Stimulierung des muskarinischen M1-Azetylcholinrezeptors führt zu einer G-Protein- und Kalzium-abhängigen Aktivierung von Calmodulin und Tyrosinphosphatasen, welche die NMDAR-Antworten wahrscheinlich über einen direkten Dephosphoryrilierungs-Vorgang hemmen. Metabotrope Rezeptoren können somit die Funktion der NMDAR über zwei entgegengesetzte Mechanismen steuern, die entweder die Tyrosinkinasen oder die Tyrosinphosphatasen aktivieren.

Im Schlusswort fasse ich neue Konzepte der metabotropen Signalisation bei der Modulierung neuronaler Aktivität zusammen. Hervorzuheben sind die unterschiedlichen, gleichzeitig durch metabotrope Stimulation aktivierten Signalkaskaden, die eher als Signalisationsnetzwerk denn als Signalisationspfad zu verstehen sind. Ferner zeigen meine Arbeiten, dass nicht nur entgegengesetzte Signalisationsmechanismen den Funktionszustand von Ionenkanälen regulieren, sondern dass auch das Kalzium als eine Art Schalter wirken kann, indem es die relative Stärke eines Transduktionsweges verändert. Ergänzend liefern meine Daten über die zellspezifische Koppelung der metabotropen Rezeptoren zu diversen intrazellulären Signalisationskaskaden in CA1 und CA3 Pyramidenzellen weitere Hinweise, dass diese zwei morphologisch zwar ähnlichen und benachbarten Zelltypen sehr unterschiedliche funktionelle Rollen im hippocampalen Schaltkreis übernehmen.

Summary

Glutamate, which is the most important neurotransmitter for excitatory synaptic signaling, acts by binding to a variety of different neuronal receptors. One of these, the N-methyl-D-aspartate receptor (NMDAR), is of particular interest because of its central role in development, synaptic plasticity, and learning and memory. In addition, disruptions in NMDAR signaling are implicated in several neurological disorders such as schizophrenia, epilepsy, Parkinson's disease, and ischemic stroke. The NMDAR belongs to the group of ionotropic glutamate receptors that also includes a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate receptors mediating fast glutamatergic neurotransmission.

NMDARs are situated mainly postsynaptically, where they form large complexes with diverse proteins (enzymes, receptors, scaffolding proteins) that constitute the transduction machinery recognized on electron microscope images as the postsynaptic density (PSD). Although NMDARs are less numerous than AMPARs and conduct only a small proportion of the postsynaptic current, they are of exceptional importance for the regulation of synaptic transmission and various other neuronal functions. A multitude of molecules and signal transduction cascades control NMDAR activity to ensure their proper functioning within a narrow physiological range. G-protein coupled (metabotropic) receptors are critical modulators of NMDAR responses through their activation of specific intracellular cascades mediating a complex interplay between metabotropic and ionotropic receptors.

The aim of my thesis project was to elucidate the role of calcium in the modulation of NMDARs by muscarinic AChRs and metabotropic glutamate receptors. During the course of this work I observed that the modulation of NMDARs is cell type specific across CA1 versus CA3 pyramidal neurons, a phenomenon that I then further characterized.

The key processes governing NMDAR modulation, with a special focus on transduction cascades initiated by metabotropic receptors, are summarized in the first chapter.

In the second chapter I present experimental data showing firstly that the direction and degree of modulation of NMDA receptors by G_q-coupled metabotropic

receptors is dependent on the extent of intracellular calcium buffering. Secondly, we found that modulation of NMDARs by GPCRs is cell-type specific such that activation of GPCRs tended to potentiate NMDA currents in CA1 pyramidal neurons but depress them in CA3 pyramidal cells. These findings may provide insights into the mechanisms underlying the selective vulnerability of CA1 versus CA3 pyramidal cells to ischemic insults.

The third chapter reports my characterization of the negative modulatory pathway mediating the muscarinic depression of NMDA currents in CA3 pyramidal neurons. Previous studies had described only a potentiation of NMDA responses in response to muscarinic stimulation. I show that stimulation of M1 mAChR subtype initiates G-protein-mediated Ca^{2+} release resulting in subsequent activation of calmodulin and a tyrosine phosphatase(s), which is likely to inhibit NMDARs by direct dephosphorylation. Thus it turns out that G_q-coupled metabotropic receptors are able to modulate NMDAR function by controlling phosphorylation state via activation of two antagonistic pathways targeting either tyrosine kinases or phosphatases.

In the conclusion I summarize current concepts on the paradigm of metabotropic signaling. Specifically, I highlight the existence of multiple, simultaneously activated, signaling cascades downstream of metabotropic receptors, which function as a "signaling network" rather than a "signaling pathway". Moreover, although the presence of parallel antagonistic pathways for the modulation of ion channels was described in the past, the present work demonstrates an additional "calcium switch" mechanism that regulates the relative gain of one pathway over the other. Finally, the cell type-specific coupling of metabotropic receptors to diverse modulatory pathways in CA1 and CA3 pyramidal neurons provides further evidence that these neighboring cell types subserve distinct functions in the hippocampal circuit.

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List of abbreviations

ACh	acetylcholine
AChR	acetylcholine receptor
AD	Alzheimer's disease
ADK	adenosine kinase
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APP	amyloid precursor protein
ASIC	acid-sensing ion channel
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BHK	baby hamster kidney cells
CaM	calmodulin
DAG	diacylglycerol
EEG	electroencephalogram
EPSC	excitotory postsynaptic current
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GFAP	glial fibrillary acidic protein
GIRK	G-protein-gated inwardly rectifying potassium current
GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
IP_3	inositol trisphosphate
KA	kainic acid
LDH	lactate dehydrogenase
LSD	lysergic acid diethylamide
LTD	long-term depression
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartic acid

NMDAR	N-methyl-D-aspartic acid receptor
NO	nitric oxide
PC	pyramidal cell
PCP	phencyclidine
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PPD	paired-pulse depression
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PSD	postsynaptic density
PTEN	phosphatase and tensin homologue
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTx	pertussis toxin
RGS	regulator of G-protein signaling
SEM	standard error of mean
SERCA	sarco/endoplasmic reticulum calcium ATPase
SFK	Src family kinases
SH	Src homology domain
STEP	striatal-enriched tyrosine phosphatase
TRPM7	transient receptor potential M7 channel

Chapter 1

Introduction

1. Introduction

1.1 The *N*-methyl-D-aspartate receptor: structure and channel properties

Early pharmacological studies characterizing the excitatory action of L-glutamate suggested a heterogeneity among glutamate receptors. In the 1970s, Jeffrey Watkins and his colleagues made a major contribution in this field by developing agonists that could distinguish between different glutamate receptor subtypes. The four agonists—*N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), kainate, and quisqualate—are distinct in the type of receptors to which they bind and have been used extensively to characterize the glutamate receptor family (Watkins et al., 1990). Quisqualate is unique in its capability of activating both ionotropic and metabotropic glutamate receptors subtypes (Hollmann and Heinemann, 1994).

Subsequent cloning of glutamate receptor subunits in 1989 in the laboratories of Stephen Heinemann, Peter Seeburg, and Richard Axel started the molecular era in glutamate receptor research (Hollmann et al., 1989; Boulter et al., 1990; Keinanen et al., 1990; Nakanishi et al., 1990). In 1990 Nakanishi and colleagues were the first to isolate the cDNA encoding for a subunit of the NMDA receptor later termed NR1 (Moriyoshi et al., 1991). At least six gene families were determined on the basis of sequence homology to encode for AMPA (one gene family), kainate (two gene families), and NMDA (three gene families) receptors (Dingledine et al., 1999). The three NMDAR gene families produce seven distinct subunits: one NR1, four NR2 (NR2A, NR2B, NR2C, NR2D), two NR3 – NR3A and the recently discovered NR3B. The NMDAR1 gene encodes a 920 amino acid polypeptide that has a molecular weight of 103 kDa. There are five hydrophobic sequences that are likely to represent the transmembrane domains, plus a long extracellular amino terminus and a shorter intracellular carboxyl terminus. Due to the NR2's larger carboxyl terminus distal to the fourth transmembrane domain (especially in NR2A and NR2B), its molecular mass is 30-63 kDa greater than NR1.

Although tremendous breakthroughs have been made in characterizing glutamate receptors, little structural data are available to date. At present, the best structurally

explored ionotropic neurotransmitter receptor is the nicotinic acetylcholine receptor (nAChR), for which electron microscopic images of near-atomic resolution have been obtained (Unwin, 2005). It was found that the native nAChR channel has a pentameric structure being assembled from five structurally similar subunits. The polypeptide chain of each subunit has four membrane-spanning domains referred to as TM1-TM4.

The subunit stoichiometry of NMDARs has not been completely elucidated and some studies propose a pentameric structure whereas others favor a tetramer (Dingledine et al., 1999). Contrary to initial expectations implicated by nAChR structural data, the transmembrane topology of the individual NMDAR subunits was found to be significantly different than for nAChRs. A current model, based on a study by (Kuner and Schoepfer, 1996), suggests that the TM2 domain of the NMDAR subunits does not completely penetrate the cell membrane but forms a re-entering loop. The transmembrane topology of NMDARs thus appears different from the four-transmembrane model of nicotinic acetylcholine receptors, but similar to that of potassium channels in that a re-entrant loop is present. Residues in this re-entrant second membrane loop are thought to control permeation properties of the ion channel (Dingledine et al., 1999).

Irrespective of stoichiometry, homogeneous NR1 subunits are not able to combine and produce functional channels in Xenopus oocytes but NR1-NR2 complexes form channels mediating NMDA-induced currents (Monyer et al., 1992; Sheng et al., 1994; Dunah et al., 1999). This effect can be explained by the finding that the glycine-binding site appears to be located on the NR1 subunit whereas the glutamate-binding domain is on the NR2 subunit. The NR1 subunit can occur in eight distinct isoforms reflecting the presence of three sites for alternative splicing (Dingledine et al., 1999). NR2A and NR2B subunits may be of particular importance for NMDAR regulated processes in forebrain structures, including the hippocampus, since their mRNA is enriched in these regions. Interestingly, during postnatal development there is a switch in NMDAR subunit expression that results in a change from NR2B to predominately NR2A expression (Monyer et al., 1992; Williams et al., 1993). The NR2C subunit mRNA is predominantly expressed in the cerebellum while NR2D expression is low in adult and is most highly expressed in the mid- and hindbrain (McBain and Mayer, 1994). Sequence analysis has revealed that there is only an 18-25% homology between the

NR1 and the NR2 family, yet within the NR2 family there is a 48-55% homology, suggesting greater similarity in structure.

The NMDAR subunit family also includes two recently characterized NR3 subunits – NR3A (also known as χ-1 and NMDAR-L) and NR3B, which are more distantly related to the other NMDAR subunits. These two subunits show a 51% similarity in sequence between each other (Nishi et al., 2001). From *in situ* and immunocytochemical analyses, NR3B is expressed predominantly in motor neurons whereas NR3A is more widely distributed (Chatterton et al., 2002). It appears that NR3A and NR3B have an important regulatory role since their co-assembly with NR1 and NR2 caused reduction in both whole-cell current (Ciabarra et al., 1995; Nishi et al., 2001) and single channel conductance (Das et al., 1998), and lowers Ca^{2+} permeability (Perez-Otano et al., 2001). These findings are supported by the fact that in NR3A knockout mice NMDA-mediated currents in cortical neurons were increased about 3-fold as was synaptic spine density (Das et al., 1998). It was recently discovered that NR3 subunits combined with NR1 impart absolutely unexpected properties to the resulting channel. Remarkably, when co-expressed in Xenopus oocytes, NR3A or NR3B co-assembles with NR1 to form excitatory glycine receptors that are unaffected by glutamate or NMDA, and inhibited by D-serine, a co-activator of conventional NMDARs (Chatterton et al., 2002). Moreover, NR1/NR3A or -3B receptors form relatively Ca^{2+}-impermeable cation channels that are resistant to Mg^{2+}, MK-801, memantine and competitive antagonists. Thus, these NR1/NR3A or -3B "NMDARs" form a type of excitatory glycine receptor. A functional receptor with this subunit composition was also found in native cerebrocortical neurons (Chatterton et al., 2002).

The biophysical properties of the NMDA receptor channel are complex (Mayer and Westbrook, 1987; Ascher and Nowak, 1988). The single-channel conductance has a main level of 50 pS; however, subconductances are evident, and different subunit combinations produce channels with distinct single-channel properties. The subunit composition of NMDARs also affects macroscopic current properties such as desensitization and deactivation. For example, NR1/NR2D receptors show no apparent desensitization in the continued presence of agonist and much slower deactivation than NR1 combined with other NR2 subunits (Dingledine et al., 1999).

The NMDA receptor channel has several unique features different from most other ligand-gated ion channels (Fig 1.1). First, along with glutamate, binding of glycine as

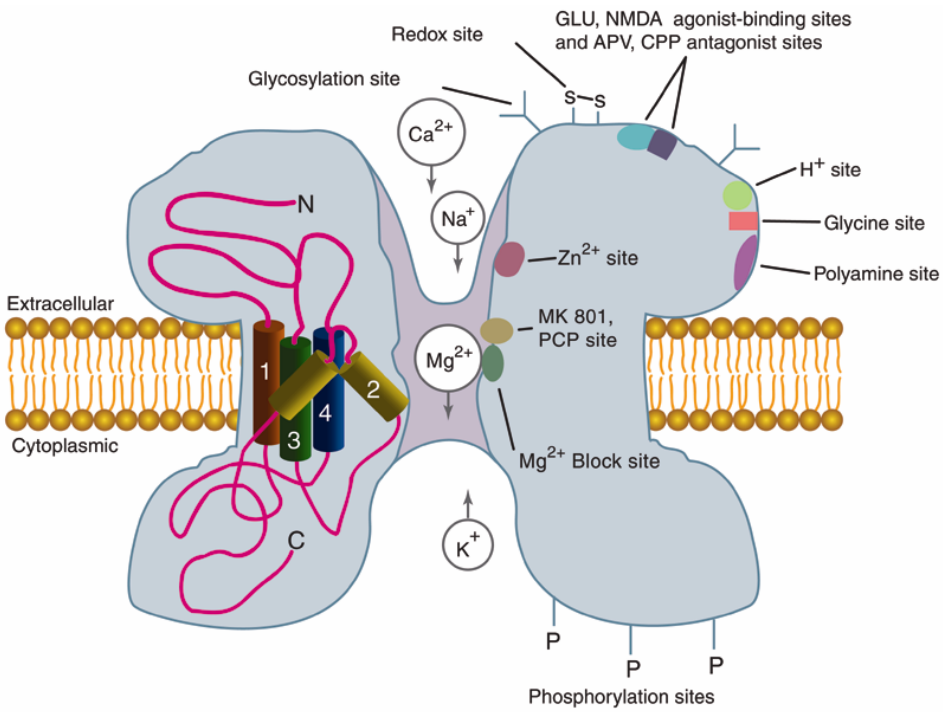

***Figure 1.1* A schematic drawing of a NMDA receptor highlighting binding sites for numerous agonists, antagonists, and other regulatory molecules.** The location of these sites is an approximation for the purpose of discussion. Adapted from (Byrne and Roberts, 2004)

a coagonist is required for effective channel opening. Glycine, which was first discovered to potentiate NMDAR activation at submicromolar levels (Johnson and Ascher, 1987), was later shown to be an essential coagonist at NMDA receptors (Kleckner and Dingledine, 1988). Second, the receptor exhibits associativity. For ions to flow, the receptor must bind glutamate and the membrane must be depolarized to remove the tonic block of the channel by extracellular Mg^{2+} at normal membrane resting potentials (Mayer and Westbrook, 1987; Ascher and Nowak, 1988). Thus the NMDA receptor is thought to serve as a "coincidence detector" being activated only after binding presynaptically released glutamate and depolarization of the postsynaptic membrane. Third, the NMDA receptor is a non-selective cation channel permeable for Na^+, K^+ and Ca^{2+} ions. Since the channel permits a significant influx of Ca^{2+}, and Ca^{2+} is a ubiquitous second messenger affecting numerous cellular functions, over-activation of NMDARs leads to excitotoxic cell death as excessive Ca^{2+} influx triggers apoptosis and necrosis. NMDA receptor function is therefore tightly controlled by numerous

signaling molecules. It has been found that the NMDA receptor is part of a large complex consisting of 77 proteins (Husi et al., 2000). Constituents of the NMDAR complex are diverse, comprising numerous kinases, phosphatases, GPCRs, scaffolding proteins, and intracellular calcium receptors. The presence of this sophisticated machinery emphasizes the importance of NMDAR regulation for normal synaptic function of neural networks.

1.2 NMDA receptors in CNS disorders

A dysfunction of glutamate receptors or their regulation is implicated in numerous neurological disorders (e.g. Parkinson disease, schizophrenia, stroke, and ischemia, and neuropathic pain). Furthermore, AMPA and NMDA antagonists tested in animal models have been shown to be protective against ischemic stroke, epilepsy, and parkinsonism symptoms. Some of the neurological disorders associated with modulation of NMDA receptors are described below. However, it should be noted that for several diseases alternative hypotheses beyond the NMDAR paradigm may be involved, which are not discussed in detail here.

1.2.1 Schizophrenia

Schizophrenia is characterized by the periodic onset of symptoms including delusions, hallucinations, paranoia, and psychosis (positive symptoms) and in most cases negative symptoms such as impaired attention and cognitive impairment. A favored theory to account for the pathophysiology of schizophrenia is the “dopamine hypothesis”, which states that overactivation of the D2 class of dopamine receptors is the molecular mechanism that underlies the behavioral abnormalities that accompany schizophrenia (for review see (Seeman and Kapur, 2000). Indeed, many of the neuroleptics effective in the treatment of schizophrenics are potent inhibitors of D2-class receptors (Van Tol et al., 1991). An alternative explanation for the etiology of schizophrenia is the “glutamate dysfunction hypothesis”, which suggests that NMDAR hypofunction accounts for many of the symptoms displayed by schizophrenics (Javitt and Zukin, 1991; Zylberman et al., 1995; Coyle, 1996; Carlsson et al., 1997; Olney et al., 1999; Lewis and Lieberman, 2000). Strong support for this theory comes from studies showing that administration of PCP, LSD, and MK-801 can induce schizophrenic-like symptoms in healthy individuals and these compounds can act, in part, as uncompetitive antagonists at NMDA receptors (MacDonald et al., 1990; Javitt and Zukin, 1991; Carlsson et al., 1997; Duncan et al., 1999; Bressan and Pilowsky, 2000). Moreover, animals expressing reduced levels of the NMDARs show behaviors similar to those seen in various animal models of schizophrenia (Mohn et al., 1999) and postmortem samples of the hippocampus from schizophrenics reveal reduced hippocampal NR1 mRNA (Law and Deakin, 2001) and NR1 protein (Gao et al., 2000).

At the same time, there are weaknesses in the "NMDA hypofunction hypothesis" of schizophrenia. For instance, NMDAR antagonists render NMDARs hypofunctional throughout the brain, whereas the experimental data suggest that in schizophrenia the defect is restricted to specific brain circuits or subcomponents. Some experts argue that the glutamate dysfunction hypothesis is consistent with the dopaminergic hypothesis of schizophrenia, as glutamate and dopamine have been reported to exhibit reciprocal actions in many regions of the CNS. Thus, a more reasonable view is that schizophrenia is a multiple neurotransmitter disease.

1.2.2 Ischemia/Stroke

Transient or persistent occlusion of cerebral blood flow results in ischemic stroke. The CA1 region of the hippocampus is particularly vulnerable to neuronal injury and ischemia-induced cell death, which is believed to result from the excessive activation of glutamate receptors (a phenomenon termed "excitotoxicity").
Over-excitation results from an increase in glutamate efflux (from presynaptic terminals and reverse glutamate transport due to Na^+ imbalance caused by intense activation of postsynaptic neurons) and a decrease in glutamate transporter activity. The excessive calcium influx via NMDARs leads to cell death, although it is becoming clear that the source of calcium and the specific pathways initiating excitotoxic cell death are more important than the general intracellular calcium load (for review see (Arundine and Tymianski, 2004).

A major disappointment in the field has been the widespread failure of clinical trials with NMDAR antagonists. Therefore the elucidation of ischemic cell death mechanisms and the search for effective therapeutic agents is still continuing. Recent studies point to two different ion channels that play crucial roles in ischemic cell death. Aarts and colleagues have proposed that TRPM7 induces anoxic neuronal death (Aarts et al., 2003) and Xiong ZG et al. have identified acid-sensing ion channels (ASICs) activated by ischemia-induced acidosis as major contributors to cell death (Xiong et al., 2004).

1.2.3 Neuropathic pain

Low doses of ketamine, an open-channel NMDAR blocker, markedly reduce chronic pain associated with spinal cord injuries (Eide et al., 1995). This observation led to the testing of further NMDAR antagonists, such as dextromethorphan, memantine, and amantadine, which also reduce perceived pain in patients (Hewitt, 2000). These findings suggest that NMDAR antagonists may represent a new class of analgesics. Clinical studies indicate that ketamine can also reduce the need for opiates in the treatment of severe pain (Wiesenfeld-Hallin, 1998). Central sensitization refers to the activity-induced changes that result in the amplification of pain. As for events related to synaptic plasticity, central sensitization involves an increase in the excitability of spinal cord neurons evoked by a cascade of responses, including neuronal depolarization to remove magnesium block of NMDAR, the resulting increase in calcium entry into neurons leading to phosphorylation of the NMDARs, and an increase in long-term synaptic strength. Consequently, processes that can lead to increased NMDAR currents (such as posttranslational modifications, trafficking of NMDARs), and stimuli that induce LTP of NMDARs are likely to by involved in pain perception.

1.3 Allosteric modulation of NMDA receptors by endogenous agents

Apart from the dependence on Mg^{2+} block and the requirement for glycine as a coagonist, NMDA receptors have been shown to be regulated by numerous additional molecules. In contrast to AMPA and kainate receptors, at least a dozen forms of allosteric modulation of NMDA receptor function by endogenous substances have been reported, which can be taken as evidence of the importance of fine-tuning of NMDA receptor function. Furthermore, many of the allosteric modulators provide tonic inhibition under physiological conditions (e.g. Mg^{2+}, H^{+}, Zn^{2+}), suggesting that allosteric modulation can protect against the deleterious consequences of NMDA receptor over-activation. Table 1 summarizes the voltage-independent regulation of NMDARs by different structurally unrelated compounds and ions.

Table 1 Endogenous extracellular voltage-independent modulation of NMDA receptor function (adapted from Dingledine, 1999)

Modulator	Effect	EC_{50}	Maximal Effect	References
Dynorphin	Inhibition	0.3 μM[b]	100%	Chen et al., 1995 a, b; Brauneis et al., 1996; Zhang et al., 1997; Chen and Huang, 1998
Osmotic pressure	Inhibition		75%	Paoletti and Ascher, 1994
Oxidizing agents	Inhibition		70%[c]	Aizenman et al., 1989, 1990, 1992; Tang and Aizenman, 1993 a,b
Protons	Inhibition	50–200 nM[d]	100%	reviewed by Traynelis, 1998
Sulfated steroids	Inhibition	150 μM[e]	100%	Park-Chung et al., 1994, 1997
Zinc	Inhibition	0.2–2 μM[g]	100–80%	Smart et al., 1994; Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998
Arachidonic acid	Potentiation	10 μM	2-fold	Miller et al., 1992; Petrou et al., 1993; Horimoto et al., 1996; Mishikawa et al., 1994; Tabuchi et al., 1997
PACAP[g]	Potentiation	<1 μM	3-fold	Liu and Madsen, 1997; Wu and Dunn, 1997
Polyamines, histamine	Potentiation	100 μM[h]	2-fold	Johnson, 1996; Williams, 1997a,b
Reducing agents	Potentiation		3-fold	Aizenman et al., 1989, 1990, 1992; Tang and Aizenman, 1993 a,b
Sulfated steroids	Potentiation	12 μM[i]	2.5-fold	Park-Chung et al., 1997

[a] Other exogenous modulators have been described: ethanol (Peoples and Weight, 1995, 1998; Masood et al., 1994; Peoples et al., 1997); ifenprodil (Legendre and Westbrook, 1991; Kew et al., 1996; Mott et al., 1998), and conanotokins (Zhou et al., 1996). Maximal inhibition (100% is full inhibition) or x-fold potentiation.
[b] Dynorphin A 1-32.
[c] The response of fully reduced receptors (e.g., dithiothreitol treated) is decreased by 70% by oxidizing agents.
[d] Proton inhibition depends on subunit composition.
[e] 3b-hydroxy-5b-pregnan-20-one sulfate.
[f] NR2A-containing receptors are much more sensitive to extracellular Zn21 than receptors containing other subunits; NR2C and NR2D are much less sensitive to Zn^{2+}.
[g] Pituitary adenylyl cyclase-activating peptide.
[h] Spermine.
[i] Pregnanalone sulfate.

1.3.1 Extracellular Zinc

In addition to its role as a cofactor in diverse biochemical pathways, zinc is also concentrated in synaptic vesicles and released in a Ca^{2+}-dependent manner at certain synapses such as the mossy fiber–CA3 pyramidal cell synapse (Vogt et al., 2000). Given this localization at CNS synapses, Zn^{2+} has attracted a great deal of attention as a possible neuromodulator of ion channels as well as a neurotoxic agent (reviewed by (Harrison and Gibbons, 1994; Smart et al., 1994; Choi and Koh, 1998; Cuajungco and Lees, 1998). It has been known for some time that group IIB transition metals such as Zn^{2+} and Cd^{2+} inhibit NMDA receptors by both a voltage-dependent and voltage-independent mechanism (reviewed by (Peters et al., 1987; Westbrook and Mayer, 1987; Mayer et al., 1989; Christine and Choi, 1990; Legendre and Westbrook, 1990; McBain and Mayer, 1994; Smart et al., 1994; Trombley and Shepherd, 1996). Zn^{2+} also inhibits glutamate uptake (Spiridon et al., 1998) and potentiates AMPA receptors (Mayer et al., 1989; Rassendren et al., 1990; Dreixler and Leonard, 1997), suggesting that Zn^{2+} release might also promote synaptic activation of non-NMDA glutamate receptors.

Several studies have shown that recombinant NMDA receptors are inhibited in a similar fashion to native receptors (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998). Voltage-dependent NMDA receptor channel blockade by Zn^{2+} is much weaker than for Mg^{2+} and appears to be qualitatively different, perhaps because of the greater permeation of Zn^{2+} than Mg^{2+} through NMDA receptors (Mayer et al., 1989; Christine and Choi, 1990; Legendre and Westbrook, 1990; Paoletti et al., 1997). Interestingly, channel block by Zn^{2+} appears to involve some of the same pore-accessible residues as channel block by Mg^{2+} (Mori et al., 1992; Kawajiri and Dingledine, 1993; Sakurada et al., 1993; Paoletti et al., 1997).

Voltage-independent Zn^{2+}-binding appears to be strongly dependent on subunit composition, and is sensitive to the type of NR2 subunits as well as the NR1 splice variants (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998). Particularly interesting is the finding that the receptors comprising the NR2A subunit are much more sensitive to Zn^{2+}, being inhibited in the nanomolar range by as much as 70 to 80% (Williams, 1996; Chen et al., 1997; Paoletti et al., 1997). Although the final concentration of Zn^{2+} particularly in the synaptic cleft remains a complex question

(reviewed by (Smart et al., 1994), it is clear that Zn^{2+} can have a multitude of effects on NMDA receptor function.

1.3.2 Reduction and oxidation of extracellular cysteine residues

Neuronal NMDA receptor function is extremely sensitive to the oxidizing potential of the extracellular environment (reviewed by (Aizenman et al., 1989; McBain and Mayer, 1994; Gozlan et al., 1995), and this redox modulation is controlled in recombinant receptors by two cysteine residues (Cys744 and Cys798) on the NR1 subunit (Sullivan et al., 1994), which are also present in the NR2 subunit (Kohr et al., 1994; Sullivan et al., 1994; Omerovic et al., 1995; Brimecombe et al., 1997). When these cysteines are oxidized by experimental reagents such as 5,59-dithiobis(2-nitrobenzoic acid), the receptor response is attenuated, and when they are reduced with compounds such as dithiothreitol the receptor response is potentiated. The reduced receptor is associated with a roughly 2-fold higher single-channel opening frequency and a slightly lower EC_{50} value for NMDA, but no change in single-channel conductance (Tang and Aizenman, 1993a; Brimecombe et al., 1997). The voltage dependence of the channel was also unchanged when receptors were treated with reducing and oxidizing agents at physiological potentials (Tang and Aizenman, 1993b). Redox modulation appears to be functionally independent of modulation of the receptor by sulfated steroids (Park-Chung et al., 1997), ethanol (Peoples et al., 1997), and pH (Traynelis and Cull-Candy, 1991; Tang and Aizenman, 1993a). It is noteworthy that the two NR1 cysteine residues that control redox modulation also control inhibition by Zn^{2+}, protons, and ifenprodil (Sullivan et al., 1994; Mott et al., 1998; Traynelis et al., 1998; Zheng et al., 1998). A wide variety of endogenous molecules has been described that are capable of oxidizing and reducing the NMDA receptor in a functionally relevant manner. These molecules include the oxidizing agents pyrroloquinoline quinone, lipoic acid, and reactive free radical oxygen species (Aizenman et al., 1990; Aizenman et al., 1992; Tang and Aizenman, 1993c; Aizenman, 1995; Scanlon et al., 1997) and reducing agents such as glutathione and dihydrolipoic acid (Gilbert et al., 1991; Manzoni et al., 1992; Tang and Aizenman, 1993b; Kohr et al., 1994; Varga et al., 1997). Nitric oxide (NO) donors also inhibit NMDARs, perhaps through the release of NO-derived compounds that support S-nitrosylation (Lipton et al., 1993; Stamler et al., 1997). However, the exact mechanism of action of NO on NMDA receptors remains

controversial (Hoyt et al., 1992; Fagni et al., 1995). Recently, additional compounds such as cyanide have been suggested to exert subunit-specific effects that appear to be linked to chemical modification through the redox site(s) of receptors containing NR2A (potentiation) or NR2B (depression; (Arden et al., 1998). Although a residual NMDA receptor response exists in the oxidized state, the difference between enhanced and oxidized responses is sufficient to suggest involvement in normal function as well as in pathological situations (Levy et al., 1990; Puka-Sundvall et al., 1995; Sinor et al., 1997). This degree of regulation of the NMDA receptor by the extracellular redox state has been considered as a potential site for therapeutic intervention in ischemic cell death (Lipton, 1993; Lipton et al., 1993). Furthermore, some compounds that oxidize the NMDA receptor and thereby reduce NMDA receptor activity are anticonvulsant and neuroprotectant in experimental models (Jensen et al., 1994; Quesada et al., 1996; Quesada et al., 1997). One important advantage of this form of modulation is the prospect of diminished side effects since oxidation does not fully inhibit the receptor. However, the feasibility of designing NMDA-specific redox modulators remains to be evaluated.

1.3.3 Extracellular Protons

The extracellular pH is highly dynamic in the mammalian CNS and influences the function of a multitude of biochemical processes and proteins, including glutamate receptor function. AMPA receptors are inhibited by protons at pH values near 6.0 rendering this effect of more biochemical than physiological interest (Christensen and Hida, 1990; Traynelis and Cull-Candy, 1990; Traynelis et al., 1995). Somatic, postsynaptic (Gottfried and Chesler, 1994; Saybasili, 1998), and presynaptic (Chen et al., 1998) native NMDA receptors are inhibited by more physiologically relevant concentrations of extracellular protons. This inhibition occurs mainly through a voltage- and agonist-independent reduction in the single-channel opening frequency rather than through changes in the single-channel open time or single-channel conductance (reviewed by (McBain and Mayer, 1994). The pH sensitivity of the NMDA receptor has received increasing attention for at least two reasons. First, the IC_{50} value for proton inhibition of exon 5- and NR2C-lacking receptors corresponds to pH 7.4, placing the receptor under tonic inhibition at physiological pH. Second, pH changes are extensively

documented in the CNS during synaptic transmission, glutamate receptor activation, glutamate receptor uptake, and also during ischemia and seizures (Siesjo, 1985; Chesler, 1990; Chesler and Kaila, 1992; Amato et al., 1994). The acidification associated with these latter pathological situations should serve to inhibit NMDA receptors, which may provide negative feedback that minimizes their contribution to neurotoxicity (reviewed by (Kaku et al., 1993; Tombaugh and Sapolsky, 1993; O'Donnell and Bickler, 1994; Munir et al., 1995; Vornov et al., 1996; Gray et al., 1997) and seizure maintenance (Balestrino and Somjen, 1988; Velisek et al., 1994). As with voltage-independent Zn^{2+} inhibition, the inhibition of NMDA receptors by protons also depends on the NR2 subunit as well as alternative exon splicing in the NR1 subunit (see (Traynelis et al., 1998). Inclusion of the NR1 exon 5 reduces both proton and Zn^{2+} inhibition, and the same amino acid residues appear to mediate both effects (see also, (Zheng et al., 1994; Traynelis et al., 1995; Traynelis et al., 1998). These structural parallels extend to other portions of the molecule in that mutations, which influence proton inhibition throughout the NR1 subunit, similarly influence Zn^{2+} inhibition ((Traynelis et al., 1998); discussed above). Interestingly, mutations that influence pH sensitivity are broad ranging in both NR1 and NR2 and include N-terminal acidic residues (Gallagher et al., 1997), cysteine residues that may participate in disulfide bond formation (Sullivan et al., 1994), residues in the extracellular M3-M4 loop (Kashiwagi et al., 1996), as well as residues that are thought to compose the channel pore-forming region (Kashiwagi et al., 1997; Traynelis et al., 1998). This latter association between pore-forming residues and pH sensitivity suggests that the proton sensor is tightly coupled to the movement of the gate.

Identification of the residues or other molecular entities that constitute the proton sensor seems an important next step, since this information might provide structural clues to NMDA receptor function and regulation. Furthermore, such information might help frame structural models describing how exon 5 of the NR1 subunit (as well as polyamines and Mg^{2+}; see below) acts as a tethered modulator to relieve tonic proton inhibition at the surface of the receptor through shielding of the proton sensor (Paoletti et al., 1995; Traynelis et al., 1995; Johnson, 1996). This information would also help to elucidate the mechanism of ifenprodil's potentiation of proton inhibition (Mott et al., 1998) and could also be useful in the design of novel NMDA receptor antagonists.

1.3.4 Extracellular Polyamines

The interactions of endogenous polyamines and polyamine toxins with ion channels have attracted considerable attention in recent years, because of both the physiological insights provided and their potential as therapeutic agents. Endogenous polyamines such as spermidine and spermine have at least three effects on NMDA receptors. Extracellular polyamines can cause voltage-dependent inhibition, glycine-dependent potentiation, and voltage- and glycine-independent potentiation of neuronal and recombinant NMDAR function (reviewed by (Rock and Macdonald, 1995; Williams, 1997a, b). The voltage-dependent block appears to involve the same intrapore residues mediating Mg^{2+} and Zn^{2+} block (Kashiwagi et al., 1997) and likely reflects fast-open channel block that is of lower affinity than Mg^{2+} with relatively weak voltage dependence (e.g., (Rock and MacDonald, 1992; Araneda et al., 1993; Benveniste and Mayer, 1993). The voltage-dependent block has a similar subunit dependence as Mg^{2+} blockade, being less pronounced for NR2Ccontaining receptors when compared with receptors containing NR2A or NR2B subunits (Williams, 1994, 1995). The structure and multivalent nature of the polyamines complicates the interpretation of blocking data in terms of a binding site at a particular location within the electric field, although it has been suggested that more than a single charge enters the electric field if the channel behaves as a single ion pore; polyamines may also permeate the channel (Benveniste and Mayer, 1993; Igarashi et al., 1997). Polyamines can stimulate NMDA receptor function to a greater degree at low glycine concentrations than at saturating glycine concentrations. This stimulation reflects approximately a 3-fold increase in glycine affinity (Benveniste and Mayer, 1993). Both glycine-independent and glycine-dependent forms of potentiation of NMDA receptor function are influenced by the NR2 subunit. However, whereas glycine-dependent potentiation occurs at NR2A- and NR2B-containing receptors, glycine-independent potentiation is observed exclusively at receptors that incorporate the NR2B subunit (Williams, 1994; Zhang et al., 1994; Williams, 1995). Consistent with the subunit selectivity, NR1 subunit mutations that influence one process do not perturb the other (Kashiwagi et al., 1996). Glycine-dependent potentiation is not controlled by NR1 RNA splicing, whereas glycine-independent potentiation is abolished when the N-terminal alternative exon 5 is incorporated into the mature transcript (Durand et al., 1993). Together, these results

suggest that two separate binding sites might exist for glycine-dependent and -independent effects of spermine. The glycine-independent form of potentiation has been suggested to arise from the relief of tonic proton inhibition at physiological pH. That is, polyamines (like the alternative exon 5) shift the pKa of the proton sensor to acidic values, reducing the degree of tonic inhibition at physiological pH, which appears as a potentiation of function (Traynelis et al., 1995).

Finally, although the physiological relevance of voltage- and glycine-independent polyamine potentiation of NMDA receptor function remains unclear given the unknown concentrations of extracellular polyamines *in vivo*, recent data have identified two endogenous activators of the polyamine site, Mg^{2+} and histamine. Mg^{2+} acts with an IC_{50} value of 2 mM to partially reduce the pH sensitivity of NR2B-containing receptors under physiological conditions (Paoletti et al., 1995). Histamine can act with an EC_{50} near 10 μM to potentiate neuronal and synaptic NMDARs (Vorobjev et al., 1993). This effect was originally suggested to involve polyamine potentiation on the basis of the nonadditive effects of spermine (Vorobjev et al., 1993). In addition, both the subunit and pH dependence of histamine potentiation support the idea that histamine potentiates NMDARs through action at the polyamine site (Saybasili et al., 1995; Yanovsky et al., 1995). Because histamine is released from widespread synaptic varicosities arising from the tuberomammillary nucleus in anterior hypothalamus, this form of regulation may be relevant under physiological conditions. Finally, aminoglycoside antibiotics may also mimic the potentiating effects of polyamines, which might contribute to the ototoxicity observed with these compounds (Segal and Skolnick, 1998). In summary, the last few years have seen the evaluation and considerable refinement of ideas about allosteric regulation in recombinant NMDA receptors. In addition to the continued discovery of new forms of regulation, one interesting trend to emerge from work on voltage-independent modulation of NMDA receptor function has been the convergence of regulatory systems. For example, site-directed mutagenesis has been used to suggest structural links between proton, Zn^{2+}, polyamine, and redox modulation of the NMDA receptor. Although it would be oversimplistic to argue that these sites are identical, there is strong evidence that they may share either partially overlapping binding determinants or common downstream structural targets.

1.4 GPCRs – G-protein coupled receptors

G-protein coupled receptors are integral membrane proteins, which are coupled to heteromeric GTP-binding proteins (G-proteins) and which are involved in the transmission of extracellular signals to the cytoplasm. Stimuli as various as neurotransmitters, hormones, odorants, light, phospholipids, and growth factors can activate specific GPCRs. These receptors participate in various physiological processes including neurotransmission, secretion, cellular metabolism, cellular differentiation, and growth as well as inflammatory and immune responses. The clinical importance of GPCRs can be exemplified by the fact that nearly a third of prescription drugs are GPCR ligands (Kotecha and MacDonald, 2003). The family of G-protein coupled receptors comprises more than 1000 members and >1% of human genes (Baldwin, 1994). GPCRs are often termed "heptahelical" receptors because of a common structural motif that consist of seven transmembrane helices, in which the N terminus is extracellular and the C terminus is intracellular, connected by alternating cytoplasmic and extracellular loops (Baldwin, 1994). The third intracellular loop is the most variable region among this group of receptors (Baldwin, 1994), which has been shown to be primarily responsible for the interaction of the receptor with G-proteins (Kobilka et al., 1998). Other intracellular segments may interact with various accessory proteins that regulate signal propagation.

1.4.1 GPCR signaling

The activation of GPCRs is thought to be a multistep process. Under basal or "resting" conditions the receptor is loosely associated with a αβγ G-protein heterotrimer with guanosine diphosphate (GDP) bound to the G_α subunit. Binding of ligand induces a profound conformational change in the transmembrane α-helices, which results in the release of GDP from the α subunit allowing guanosine triphosphate-magnesium (GTP-Mg^{2+}) to bind (Hamm and Gilchrist, 1996; Freissmuth et al., 1999). The activated G_α subunit has weaker affinity to $G_{\beta\gamma}$, which leads to a dissociation allowing the G_α and $G_{\beta\gamma}$ subunits to interact and to activate appropriate downstream signaling cascades. Deactivation and return to the basal state occurs when the intrinsic GTPase activity of

the α subunit cleaves the terminal phosphate group of GTP such that GDP-liganded G_α reassociates with $G_{\beta\gamma}$. This process can be accelerated by interaction with an effector system or by binding of regulator of G-protein signaling proteins (RGSs) (Pitcher et al., 1998; De Vries et al., 2000).

In mammals there are 20 G_α subnits, 6 G_β subunits, and 12 G_γ subunits that theoretically provide 1440 combinatorial signal transduction possibilities (Clapham and Neer, 1997). The G_α subunits can be subdivided into four families according to their primary sequence similarity: G_s, G_i, G_q, and G_{12}. These subunits are pivotal to the regulation of several second-messenger-generating systems. For example, the G_q family regulates the activity of phosphatidylinositol-specific phospholipases, such as PLC; the members of the G_s family activate adenylyl cyclases, whereas G_i inhibits a subset of these enzymes. Although the $G_{\beta\gamma}$ subunit consists of two polypeptides functionally it behaves as a monomer. Initially $G_{\beta\gamma}$ was thought to simply regulate G_α activity, but now it is clear that $G_{\beta\gamma}$ acts as a signal transduction molecule in its own right. To date $G_{\beta\gamma}$ has been shown to regulate just as many different protein targets as the G_α subunit (for review see (Clapham and Neer, 1997; Betty et al., 1998; Ford et al., 1998; Brunk et al., 1999). One of the interesting actions of the $G_{\beta\gamma}$ subunit is the direct activation of certain ion channels, such as the G-protein-gated inward rectifying K^+ (GIRK) channels (Reuveny et al., 1994; Krapivinsky et al., 1995).

Although the very name “G-protein coupled receptor” implies involvement of the heteromeric GTP-binding proteins in the GPCRs signaling, the textbook paradigm of metabotropic signal transduction is slowly changing in light of recent studies suggesting that GPCRs can also transduce signals without G-protein activation. For example, it has been shown that activation of the nonselective cation current induced by mGluRs and mAchRs in rat hippocampus depends on the activation of a G-protein independent pathway (Guerineau et al., 1995; Gee et al., 2003) and the modulation of NMDARs by mGluR1 in CA3 pyramidal cells of the hippocampus relies on a G-protein independent mode of signaling (Benquet et al., 2002). The feasibility of G-protein independent signaling is supported by structural and biochemical data showing a close association of diverse enzymes and adaptor proteins with GPCRs thus providing an alternative mechanism for the transduction of extracellular stimuli into the cellular interior (for review see (Heuss and Gerber, 2000).

1.4.2 Muscarinic receptors in the hippocampus

The cholinergic system is considered to be one of the most important neuromodulatory (as opposed to executive) neurotransmitter systems in the brain. Cholinergic neurons are distributed in a variety of different nuclei of which two groups in the basal forebrain and pedunculopontine area are especially prominent and project extensively to the cortex and thalamus (Fig. 1.2) (Perry et al., 1999). In the hippocampus one of the major modulatory inputs is the cholinergic projection originating in the medial septum and the diagonal band of Broca. However, the low number of identifiable synaptic-membrane differentiations apposing choline acetyltransferase immunostained axon terminals in the rat cortex and hippocampus (Descarries et al., 1997), indicates that both "diffuse" as well as synaptic point-to-point ACh-mediated transmission may be important.

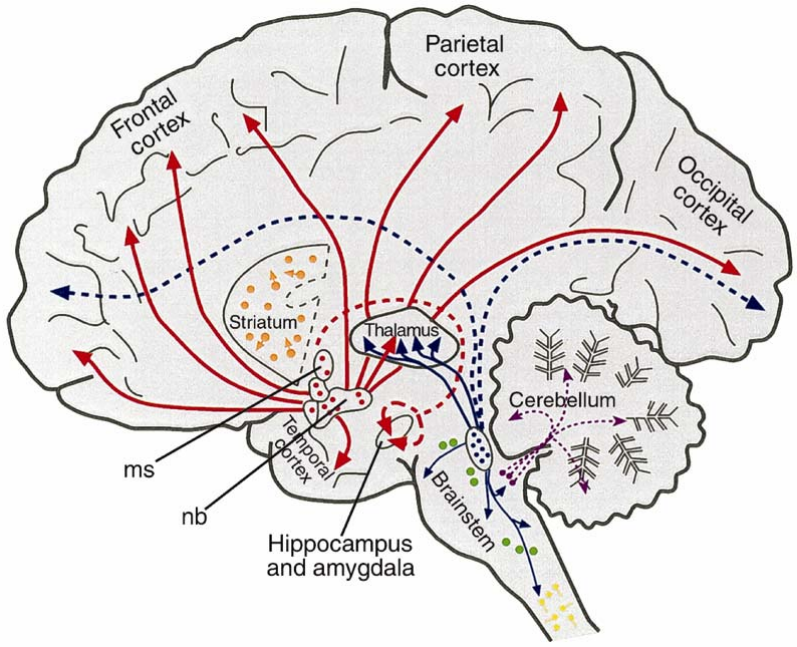

***Figure. 1.2*. Cholinergic systems in the human brain.** Two major pathways project widely to different brain areas: basal-forebrain cholinergic neurons [red, including the nucleus basalis (nb) and medial septal nucleus (ms)] and pedunculopontine–lateral dorsal tegmental neurons (blue). Other cholinergic neurons include striatal interneurons (orange), cranial-nerve nuclei (green circles), vestibular nuclei (purple); and spinal-cord preganglionic and motoneurons (yellow). The habenula–interpeduncular pathway is not shown. Adapted from (Perry et al., 1999)

Two different classes of receptors mediate effects of acetylcholine: muscarinic receptors (mAChRs), which are large transmembrane GPCRs (Hulme et al., 1991), and nicotinic receptors (nAChRs), which form nonselective cation channels (Dani and Mayer, 1995; Boyd, 1997). Like other GPCRs, muscarine receptors contain seven transmembrane domains and are associated with G-protein subunits. Immunohistochemical studies have shown that M1, M3, and M4 are major mAChR subtypes in hippocampal pyramidal neurons (Levey et al., 1995).

Historically, the first observations indicative of multiple muscarinic receptor subtypes were the cardioselective actions of gallamine (Riker and Wescoe, 1951) and the sympathetic ganglionic stimulating behavior of (4-hydroxy-2-butynyl)-1-trimethylammonium-m-chlorocarbanilate chloride (McN-A-343) (Roszkowski, 1961). Subsequently it was shown that various compounds show a clear difference in affinity to muscarinic receptors in atrial pacemaker cells compared to ileum (Barlow et al., 1976) which suggested the existence of at least two different mAChRs subtypes.

Cloning of cDNAs for muscarinic receptor genes was pioneered by the work of Numa and colleagues, who cloned the M1 and M2 genes (Kubo et al., 1986a; Kubo et al., 1986b), and was extended by the discovery of the M3, M4, and M5 genes (Bonner et al., 1987; Peralta et al., 1987; Bonner et al., 1988). These five genes encode the muscarinic receptor proteins (more precisely, glycoproteins), which have the structural features of the seven transmembrane helix G-protein-coupled receptor family. Muscarinic receptor sequences have significant homologies with other members of this receptor superfamily (Hulme et al., 1990).

It is well established that the “odd-numbered” muscarinic receptors (M1, M3, M5) typically couple via the α subunits of the $G_{q/11}$ family, whereas the “even-numbered” members (M2, M4) couple to their effectors via G_i and G_o α subunits. An important molecular distinction between the different muscarinic receptor subtypes is the sequence divergence in the postulated third internal (i3) loops between the M1/M3/M5 sequences compared with the M2/M4 sequences (Hulme et al., 1990; Wess, 1996; Wess et al., 1997) that probably determines the fairly specific coupling preferences of these two groups (Wess, 1993). Coupling selectivity at the G-protein level translates in general to specific targeting to downstream second-messenger pathways activated by the two groups of muscarinic receptors with phospholipase C_β being activated by the “odd” receptors (M1/M3/M5), whereas adenylyl cyclase is

inhibited by the "even" receptors (M2/M4) (reviewed by (Caulfield, 1993; Felder, 1995).

Interestingly, cholinergic modulation is implicated in the regulation of cognitive function and its impairment in certain CNS disorders. For instance, a disruption in cholinergic neurotransmission involving mAChRs appears to contribute to the dementia symptoms in Alzheimer disease (AD). Thus, AD patients show: (i) a consistent depletion of choline acetyltransferase in neocortex and hippocampus (Perry et al., 1978; Coyle et al., 1983); (ii), a reduction in the number of cholinergic basal forebrain neurons (Whitehouse et al., 1981; Arendt et al., 1983); (iii) a correlation of choline acetyltransferase levels (Perry et al., 1978; Coyle et al., 1983) and numbers of basal forebrain neurons (Doucette et al., 1986; Lehericy et al., 1993) with the severity of dementia. In addition, lesions of basal forebrain neurons and pharmacological blockade of mAChR are related to cognition deficits in animal studies (Dunnett, 1985; Nilsson et al., 1992) and humans (Drachman and Leavitt, 1974; Damasio et al., 1985). Furthermore, restoration of function in lesioned animals has been demonstrated upon implantation of ACh-secreting cells (Nilsson et al., 1992; Winkler et al., 1995). Stimulation of mAChRs also selectively influences the processing of the amyloid precursor protein (APP), such that receptor activation increases the secretion of non-amyloidogenic peptides both *in vitro* (Farber et al., 1995) and *in vivo* (Lin et al., 1999). Although the muscarinic hypothesis of AD is under debate, clinical trials with acetylcholinesterase inhibitors such as tacrine (Cognex), donepezil (Aricept) and rivastigmine (Exelon) have shown some amelioration of certain cognitive deficits (Perry et al., 1999).

Knockout studies have demonstrated that mice devoid of one or several mAChR subtypes show behavioral deficits and altered electrophysiological, and biochemical properties at the cellular level. For example M1 knockout mice exhibit increased locomotor activity (Gerber et al., 2001; Miyakawa et al., 2001), lack of muscarine-mediated oscillations in area CA3 of the hippocampus (Fisahn et al., 2002), lack of pilocarpine-mediated seizure activity and a loss of muscarinic agonist-mediated M current (I_m) inhibition (Hamilton et al., 1997).

1.4.3 Activation of M1 receptors potentiates NMDA-mediated responses

Henry Markram and Menahem Segal were the first to discover that ACh potentiates NMDA responses (Markram and Segal, 1990a) and their experiments in CA1 pyramidal neurons indicate that the ACh-induced potentiation is mediated via mAChR receptors (Markram and Segal, 1990b). Activation of mAChRs in CA1 potentiates NMDARs both in acute slices (Markram and Segal, 1990b; Marino et al., 1998) and in dissociated cells (Lu et al., 1999) as well as in the other cell types such as striatal spiny neurons (Calabresi et al., 1998), and in auditory neocortical cells (Aramakis et al., 1999). The muscarinic receptor subtype mediating the potentiation is likely M1 as specific M1 toxins blocked the carbachol-induced potentiation (Marino et al., 1998). Consistent with this finding, M1 receptors were also shown to co-localize with NR1A at specific postsynaptic sites (Marino et al., 1998) and M1 muscarinic receptors are highly expressed in the hippocampus (Levey et al., 1995). The mechanism underlying the potentiation long remained elusive. Initially Markram and Segal reported that the M1-induced enhancement of NMDA responses required activation of PI turnover via G_q subunits, however, the downstream signaling molecules were not identified (Markram and Segal, 1992). Later it was suggested that PKC was involved (Calabresi et al., 1998) given that NMDAR currents were enhanced in response to application of phorbol esters, yet others failed to confirm this finding (Harvey et al., 1993). The activation of muscarinic receptors or PKC is also known to activate tyrosine kinases in hippocampal slices (Stratton et al., 1989). In 1999 John MacDonald and colleagues showed that in the CA1 region endogenous PKC activates the nonreceptor tyrosine kinase Src to enhance NMDA responses, and application of muscarine enhanced NMDA channel activity via a PKC/Src cascade (Lu et al., 1999). Pyk2, a focal adhesion kinase, was identified as the intermediate protein linking activation of PKC to activation of Src (Huang et al., 2001), hence M1 receptors activate a PKC/Pyk2/Src cascade that results in the enhancement of NMDAR- mediated currents.

1.4.4 Metabotropic glutamate receptors

Data from early studies suggested that only extrinsic afferents to the hippocampus, releasing dopamine, acetylcholine, serotonin, or norepinephrine, modulated ionotropic glutamate receptor (AMPA, NMDA, and kainate) currents. However, in the mid-1980s it became evident that glutamate could stimulate PI turnover or lead to the mobilization of intracellular calcium (Sladeczek et al., 1985; Nicoletti et al., 1986; Pearce et al., 1986; Sugiyama et al., 1987; Mayer and Miller, 1990). The receptors that mediate this response were termed metabotropic glutamate receptors (mGluRs) as they modulated cellular metabolism and displayed distinct functional and pharmacological properties from ionotropic glutamate receptors. The metabotropic glutamate receptors (mGluRs) are similar in general structure (in having seven transmembrane-spanning segments) to other GPCRs but are divergent enough to be considered to have originated from a separate evolutionary derived receptor family (Hollmann and Heinemann, 1994; Nakanishi, 1994). In fact, sequence homology between the mGluR family and the other G-protein-linked, seven transmembrane-segment receptors is minimal. The mGluR family is heterogeneous in size, ranging from 854 to 1179 amino acids. Both the N-terminal and C-terminal domains are unusually large for GPCRs. A major difference in the structure of mGluRs is that the agonist binding site resides in the large N-terminal extracellular domain and is homologous to a bacterial amino acid-binding protein (O'Hara et al., 1993). In most of the other families of metabotropic receptors, the ligand-binding pocket is formed by the transmembrane segments partly buried in the membrane. This significant structural distinction supports the idea that the mGluRs evolved separately from the other metabotropic receptors. Also the second intracellular loop plays a critical role in G-protein-coupling specificity, rather than the third as with most of the other GPCRs, (Conn and Pin, 1997).

Based on structural homology, pharmacology, agonist selectivity, and signal transduction mechanisms the eight members of mGluR (mGluR1-8) have been classified into group I, II, or III. Group I consist of mGluR1 and mGluR5, group II of mGluR2-3, and the rest belong to group III. mGluR1 has variants, termed mGluR1α, β, c, d, and e, which are produced by alternative splicing. Isoforms β, c, and d lack the long carboxyl terminus specific for mGluRα. Transfection experiments in *Xenopus* oocytes or baby hamster kidney (BHK) cells have revealed that all mGluR1 splice variants activate PLC, yet there is a difference in agonist sensitivity (Flor et al., 1996). mGluR5 has two splice variants termed mGluR5a and mGluR5b. Unlike mGluR5a,

mGluR5b contains an insertion of 32 amino acids after the seventh transmembrane domain (Conn and Pin, 1997). It is likely that this region imparts unique signal transduction properties. In almost every cell system studied, group I mGluRs stimulate PLC and PI hydrolysis (for review see (Conn and Pin, 1997), although in some cases these receptors are linked to adenylyl cyclase activity (Aramori and Nakanishi, 1992). In the case of mGluR5, no sensitivity to pertussis toxin (PTx) was observed suggesting that these GPCRs may be linked to G_q-like proteins, which have conventionally been linked to activation of PLC (Abe et al., 1992; Pickering et al., 1993). Indeed, activation of mGluR5 is positively coupled to PLCβ1 in neurons (Hannan et al., 2001). The mGluR agonist t-ACPD is also able to stimulate phospholipase D (PLD) in adult and neonatal hippocampal slices (Boss et al., 1992; Holler et al., 1993), although at least one group suggests that this may be mediated by a non-mGluR group I receptor (Pellegrini-Giampietro et al., 1996). Coupling mechanisms of group II and III are less explored but are thought to induce cellular responses largely via inhibition of cAMP production.

Immunocytochemical studies have shown that the mGluR1α splice variant is expressed in the caudate putamen, the cerebral cortex, the amygdaloid complex, and the hippocampus (Ferraguti et al., 1998). Within the hippocampus mGLR1α is localized to non-principal neurons of the CA1 region and low expression is detected in CA1 pyramidal neurons (Baude et al., 1993; Ferraguti et al., 1998). Interestingly, the mGluR1β isoform is not expressed in the CA1 region of rodents and isoform c expression is restricted to the dentate granule cells (Ferraguti et al., 1998). On the other hand, mGluR5 is richly expressed in pyramidal neurons in the CA1 area on both peri- and extrasynaptic sites (Lujan et al., 1996).

Synaptic transmission is affected by mGluRs via modulation of postsynaptic function as well as modulation of transmitter release from presynaptic terminals. The actions on presynaptic boutons are largely due to activation of group II and III mGluRs (Baskys and Malenka, 1991; Glaum and Miller, 1994) for review). Interestingly, several recent studies suggest that a lattice of scaffolding proteins link group I mGluRs to IP_3 receptors (Tu et al., 1999) and mGluR5 and mGluR1 are linked to NMDARs via a Homer/Shank/GKAP/PSD-95 complex of proteins (Brakeman et al., 1997; Naisbitt et al., 1997; Naisbitt et al., 1999; Roche et al., 1999; Tu et al., 1999; Naisbitt et al., 2000). This finding may indicate a potential role for mGluRs in modulation of synaptic transmission.

1.4.5 Group I mGluRs modulate NMDAR function

Group I metabotropic glutamate receptors are located perisynaptically in close proximity to NMDARs, which suggests a possible modulatory influence of mGluRs on NMDA receptor function (Baude et al., 1993; Lujan et al., 1996; Lujan et al., 1997). The effect of mGluR activation on NMDA responses was investigated by many groups, however, their results were often contradictory even as to whether mGluRs potentiate NMDA receptor function (Aniksztejn et al., 1991; Bleakman et al., 1992; Harvey and Collingridge, 1993) (for review, see (Anwyl, 1999; Valenti et al., 2002) or inhibit NMDA-mediated responses (Yu et al., 1997a; Wang et al., 1998; Zhong et al., 2000; Snyder et al., 2001). It was found that mGluRs modulating NMDA receptor function belong to group I, either mGluR1 (Lan et al., 2001; Skeberdis et al., 2001; Heidinger et al., 2002) or mGuR5 (Doherty et al., 1997; Jia et al., 1998; Awad et al., 2000; Mannaioni et al., 2001; Pisani et al., 2001). Another discrepancy in these studies was with respect to the mechanisms transducing the effect of mGluRs on NMDARs. For instance, several earlier studies suggested participation of a PKC-dependent pathway in the mGluR-dependent potentiation of NMDA responses (Aniksztejn et al., 1991; Kelso et al., 1992; Pisani et al., 1997; Ugolini et al., 1997; Skeberdis et al., 2001) but other investigators reported a PKC-independent process (Harvey et al., 1993; Kinney and Slater, 1993; Rahman and Neuman, 1996; Holohean et al., 1999). The comprehensive study work by Pascal Benquet and colleagues has in part reconciled the earlier data showing that in CA3 hippocampal neurons the effects of the two group I mGluRs converge on the nonreceptor tyrosine kinase Src. In this report mGluR1 was shown to activate Src via a G-protein independent (and hence PKC independent, as well) mechanism and mGluR5 stimulated the “conventional” G-protein/PLC/PKC/Src pathway (Benquet et al., 2002). Furthermore, Ca^{2+} was found to act as a negative-feedback element in the modulation of NMDARs, whereas other studies showed that Ca^{2+} is indispensable for the mGluR-induced potentiation of NMDAR responses (Skeberdis et al., 2001). Thus, in CA1 pyramidal cells co-activation of mGluR5 and NMDARs potentiates NMDAR currents in a Ca^{2+}-dependent manner, whereas activation of mGluR5 alone fails to enhance NMDAR responses, producing a slight depression instead (Kotecha et al., 2003). This finding suggests that activation of

NMDARs acts to “load” intracellular calcium stores, which are required for the mGluR5-induced enhancement of NMDA current. In conclusion, it is evident that both branches of G_q-mediatied signaling (activation of PKC cascades and Ca^{2+} release) as well as a G-protein independent pathway contribute to the mGluR-induced modulation of NMDAR activity.

1.5 Src family kinases and the regulation of NMDA receptors

Src, the first discovered member of a family of nonreceptor protein kinases, was initially identified as the transforming protein (v-Src) of the oncogenic retrovirus Rous sarcoma virus (Bishop, 1983). Subsequently it was discovered that the cellular homolog of v-Src possesses tyrosine kinase activity (Bishop, 1983). At present eight members belonging to the Src family kinases (SFKs) are known, comprising Fyn, Yrk, Hck, Lyn, Blk, Yes, Fgr, and Lck. The SFKs mediate a broad spectrum of physiological responses including cell cycle control, proliferation, differentiation, adhesion, migration and survival (Thomas and Brugge, 1997). The SFKs range in size from 52 to 62 kDa and share a similar domain structure consisting of an amino terminus, a "unique" domain, the catalytic domain or Src homology (SH1) domain 1, the SH2, SH3 domains and a carboxyl terminus (Salter and Kalia, 2004) (Fig. 1.3). Src kinase can be activated via dephosphorylation of the tail or competition for the SH2 or SH3 domain. Once activated SFKs transfer the γ-phosphate of ATP to tyrosine residues on protein substrates.

Five of the SFKs are highly expressed in the mammalian CNS – Src, Fyn, Yes, Lck and Lyn. During development of the CNS they are involved in neuronal differentiation and neurite outgrowth (Kuo et al., 1997; Hoffman-Kim et al., 2002). However, in recent years it has become clear that in differentiated postmitotic cells of the adult CNS these kinases fulfill distinct functions. SFKs play a critical role in the regulation of various voltage-gated ion channels such as potassium (Fadool et al., 1997) and calcium channels (Cataldi et al., 1996) as well as ionotropic neurotransmitter receptors including γ-aminobutyric acid type A ($GABA_A$) receptors (Moss et al., 1995; Wan et al., 1997) and nicotinic acetylcholine (nAChR) receptors (Wang et al., 2004). The first ion channel shown to be modulated by Src was the NMDAR. Wang and Salter characterized the effects of tyrosine phosphorylation and dephosphorylation in the modulation of NMDARs using electrophysiological recordings and pharmacological blockade of PTKs and PTPs (Wang and Salter, 1994). They showed that NMDAR function is dependent on a balance between the activity of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs): inhibiting

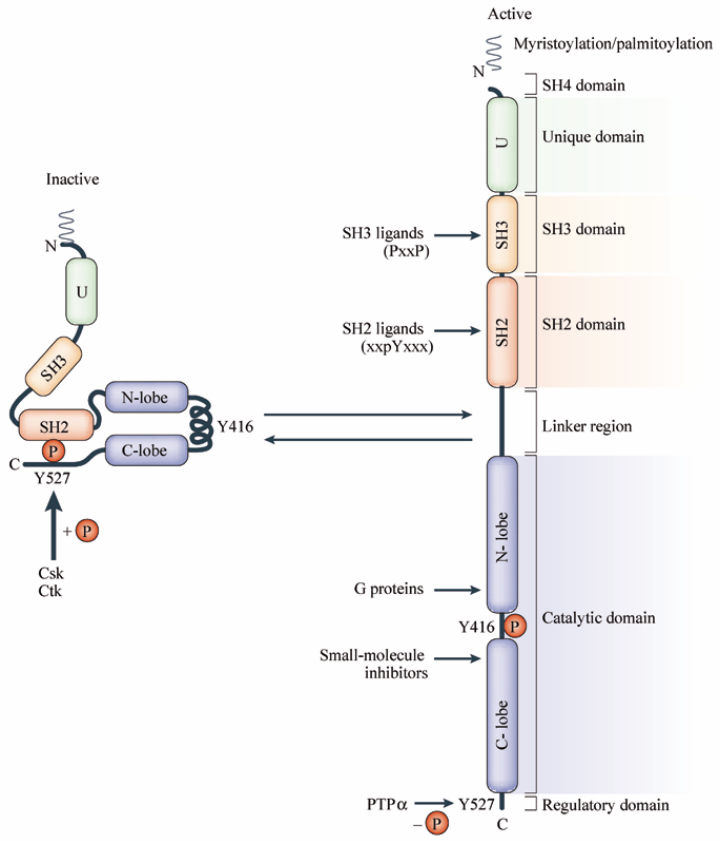

Figure 1.3. **Structure and regulation of Src family kinases (SFKs).** Members of the Src family of protein tyrosine kinases share a common domain structure that includes the Src homolgy 4 (SH4) domain, unique domain (U), SH3 domain, SH2 domain, linker region, catalytic domain (SH1 domain, N- and C-lobes) and regulatory domain. The regulation of SFK catalytic activity is mediated by intramolecular interactions and tyrosine phosphorylation (P) or dephosphorylation of the kinase itself. Left, in the inactive conformation, the SH2 domain interacts with phosphorylated Y527 in the regulatory domain, the SH3 domain interacts with a ligand in the linker region, and Y416 in the activation loop is dephosphorylated. The inactive conformation is supported by the activity of Csk or Ctk, which phosphorylates Y527. Right, displacement of the intramolecular interactions by binding of SH2 and/or SH3 domain ligands and by dephosphorylation of Y527 by protein tyrosine phosphatases (such as PTP) leads to the active conformation. Autophosphorylation of Y416 results in a conformational change of the activation loop, which renders the kinase fully active. Small-molecule inhibitors (such as PP2) bind to the ATP-binding site in the catalytic domain and block phosphoryl transfer to target proteins. Some G proteins (such as $G_{\alpha s}$, $G_{\alpha i}$ and H-Ras) interact with the catalytic domain and alter kinase activity by unknown mechanisms. By convention, the amino-acid residue numbers shown are relative to chicken Src. Adapted from (Salter and Kalia, 2004)

endogenous PTK activity (Wang and Salter, 1994; Wang et al., 1996) or introducing exogenous PTP (Wang et al., 1996) suppresses NMDAR currents, whereas inhibiting endogenous PTP activity or introducing exogenous Src enhances NMDAR currents (Wang and Salter, 1994). Activation of SFKs also potentiates NMDA responses in heterologous cells where exogenous Src or Fyn potentiates currents mediated by recombinant NMDARs expressed in HEK293 cells (Kohr and Seeburg, 1996) or in

Xenopus oocytes (Chen and Leonard, 1996). In *Xenopus* oocytes the Src-mediated enhancement of NMDAR currents requires co-expression of PSD-95 (Liao et al., 2000), reflecting the fact that Src is physically associated with the NMDAR complex, which brings together the kinase and its respective substrates (Yu et al., 1997b; Husi et al., 2000). Single channel recordings demonstrated that Src potentiates NMDA responses by increasing the open probability of the channel without changing single channel conductance (Wang et al., 1996). The potentiation by Src appeared to affect only peak NMDA current but not steady-state current (Lu et al., 2000).

Interestingly, inhibition of PTKs or PTPs in excised patches affected NMDARs in the same way (Wang et al., 1996), which suggests a close association of tyrosine kinases and phosphatases within the NMDAR complex.

The involvement of SFKs in the enhancement of NMDAR function was established by using SFK-specific pharmacological tools: a phosphopeptide SFK activator (pYEEI peptide), which is a ligand for SFK SH2 domains, and a function-blocking antibody (anti-cst1), which inhibits SFKs but not other PTKs (Roche et al., 1995). Nevertheless, it is still not clear whether all five or only specific members of the SFKs expressed in the CNS are responsible for the upregulation of NMDAR function. Src (Huang et al., 2001) and Fyn (Suzuki and Okumura-Noji, 1995), as well as Lck, Lyn, and Yes (Kalia and Salter, 2003), are found in the postsynaptic density. Moreover, Src (Yu et al., 1997b) and Fyn (Yaka et al., 2002), as well as Lyn and Yes (Kalia and Salter, 2003), were shown to be components of the NMDAR complex. Thus, Src, Fyn, Lyn, and Yes are all strategically positioned within the PSD to potentially regulate NMDAR function. Participation of Src itself in the potentiation of NMDAR function was established through the use of Src-specific agents – an inhibitory antibody (anti src1) (Roche et al., 1995) and an inhibitory peptide (Src 40-58) (Yu et al., 1997b) that selectively blocks Src but not other SFKs. Each of these Src-specific inhibitors depressed NMDA-mediated currents and decreased NMDAR channel gating – the same changes produced by the general inhibitor, the anti-cst1 antibody. As mentioned above, in cell expression systems NMDA-mediated currents were shown to increase when exogenous Fyn was included in the patch pipette solution (Kohr and Seeburg, 1996), but whether endogenous Fyn, or other SFKs present in the CNS, modulates native NMDARs is still not clear because of the lack of selective antagonists blocking a particular member of SFKs. Src, however, is thought to be critical in the upregulation of

NMDAR function since Src-specific inhibitors prevent the increase in channel activity produced by the SFK-activating pYEEI peptide (Yu et al., 1997b).

The precise mechanism through which SFKs upregulate NMDAR function is not known. The NR2B subunit of NMDARs has been identified as a major tyrosine-phosphorylated protein in the postsynaptic density (Moon et al., 1994). NR2A was also found to be phosphorylated on tyrosine, whereas the NR1 subunit seems not to be tyrosine phosphorylated (Lau and Huganir, 1995). These findings raise the question whether phosphorylation of one or more tyrosine residues in NR2A or NR2B underlies the increase of NMDAR currents. But even though phosphorylation of several tyrosine residues has been demonstrated, whether receptor phosphorylation induces the upregulation of NMDAR function remains unknown. The NR2A and NR2B subunits have large C-terminus tails containing 25 tyrosine residues each . By means of site-directed mutagenesis, Y1292, Y1325 and Y1387 were identified as sites for Src-mediated phosphorylation in the NR2A C-tail (Yang and Leonard, 2001) and Y1252, Y1336 and Y1472 in the NR2B C-tail as sites of Fyn-mediated phosphorylation (Nakazawa et al., 2001). In recombinant NR2B subunits, Y1472 is the main site of phosphorylation (Nakazawa et al., 2001).However, the question remains whether phosphorylation of these residues underlies the Src-mediated increase in NMDAR channel open probability. The simplest way to arrive at an answer would be to mutate the specific tyrosine residues in the C-terminus tail, which should block Src-mediated upregulation of NMDAR currents. Until now there are no such studies for the NR2B subunit and there is the only one for NR2A. In this study the authors found that phosphorylation of three C-terminus tyrosine residues of NR2A by Src modulates the sensitivity of NMDARs to tonic inhibition by Zn^{2+} (Zheng et al., 1998). However, a subsequent study revealed that in native hippocampal and spinal neurons Src does not potentiate NMDAR currents by removing Zn^{2+} inhibition (Xiong et al., 1999) and therefore the above-mentioned phenomenon can be attributed to an artifact associated with the cell expression system.

Thus, despite the fact that Src-mediated phosphorylation of several tyrosine residues on the NR2 subunits has been firmly established, a direct relation between NR2 phosphorylation and a change in NMDAR gating has not been shown, leaving open the possibility that other proteins in the NMDAR complex mediate the Src-induced potentiation.

1.5.1 $STEP_{61}$ in opposition to Src

Tyrosine phosphorylation, a type of posttranslational protein modification, has been recognized as a dynamic process governed by the opposing activities of protein tyrosine kinases and protein tyrosine phosphatases (Paul and Lombroso, 2003). According to this concept there must exist a PTP that counteracts Src-mediated phosphorylation of tyrosine residues on NMDARs. This prediction is supported by earlier findings on the role of PTKs and PTPs in the modulation of NMDARs where inhibition of PTPs in excised membrane patches increased NMDAR channel gating (Wang et al., 1996) and PTP activity co-immunoprecipitated with NMDARs (Ali and Salter, 2001). These experimental results indicate that an endogenous PTP is an integral part of the NMDAR complex. Although several PTPs have been shown to be a part of the NMDAR complex or, at least, to be localized to the postsynaptic density (e.g. (Ning et al., 2004), a PTP fulfilling the criteria for an "anti-Src PTP" was only recently identified (Pelkey et al., 2002). Striatal-enriched phosphotase (STEP) located at the PSD of glutamatergic synapses (Oyama et al., 1995) is a member of a family of brain-specific, nonreceptor type intracellular PTPs (Paul and Lombroso, 2003). STEP belongs to a group of PTPs that currently has three members expressed in vertebrates (Lombroso et al., 1991; Hendriks et al., 1995; Ogata et al., 1995). This brain-specific phosphatase is preferentially expressed in neurons of the basal ganglia, hippocampus, cortex and related structures (Lombroso et al., 1993; Boulanger et al., 1995). STEP family members are produced by alternative splicing, and both cytosolic ($STEP_{46}$) and membrane-associated ($STEP_{61}$) variants exist (Bult et al., 1996; Bult et al., 1997). Furthermore, some STEP members are truncated isoforms that lack the catalytic phosphatase domain (Sharma et al., 1995). The functions of the truncated isoforms are not known, although they may serve analogous functions to the tyrosine kinases that exist as truncated isoforms and bind to substrates to protect them from phosphorylation. The $STEP_{61}$ isoform is part of the NMDAR complex in the spinal cord and hippocampus (Pelkey et al., 2002) and, therefore, is appropriately positioned to counteract Src and downregulate NMDAR function. Functional studies have shown that application of recombinant STEP to the cytoplasmic side of a membrane patch decreases NMDAR channel gating resembling the effect of blocking Src. Recombinant STEP applied intracellularly decreased synaptic NMDAR currents as well (Pelkey et al.,

2002). Conversely, intracellular application of an anti-STEP antibody or a dominant-negative STEP increased synaptic NMDAR currents implying that NMDAR function is regulated by endogenous STEP. Both the reduction of NMDAR currents resulting from the application of exogenous STEP and the increase of NMDAR currents produced by blocking endogenous STEP required Src, as both were prevented by blocking Src activity (Pelkey et al., 2002) (see Fig 1.4). Interestingly, the activity of STEP is controlled by different neurotransmitter-controlled signaling cascades so that stimulation of dopamine D1 receptors phosphorylates and inactivates STEP via a PKA-dependent pathway (Paul et al., 2000) whereas NMDA-mediated Ca^{2+} influx leads to calcineurin-dependent dephosphorylation and activation of STEP (Paul et al., 2003). Thus STEP acts as a PTP that counterbalances Src-mediated tyrosine phosphorylation of NMDARs. However, in contrast to our knowledge about the numerous pathways converging on SFKs, the physiological stimuli leading to the activation of STEP and the underlying signal transduction cascades remain poorly understood.

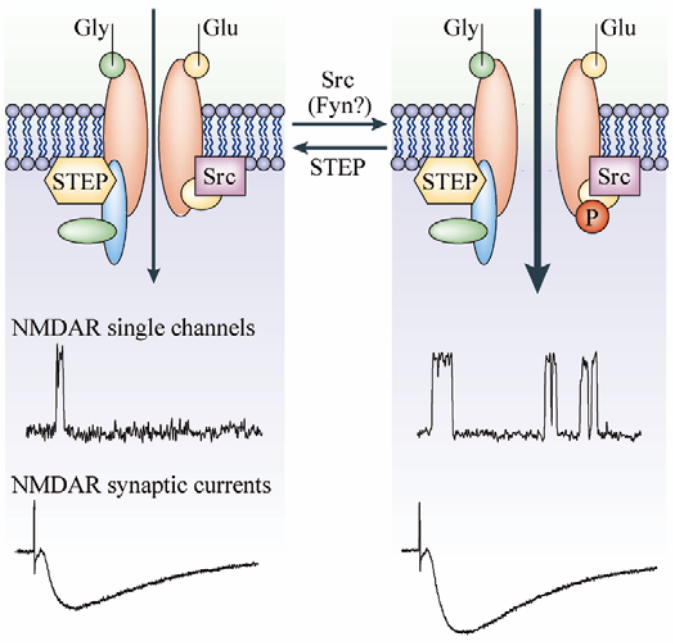

Figure 1.4 **Regulation of NMDAR gating by the balance of tyrosine phosphorylation and dephosphorylation.** STEP (striatal enriched tyrosine phosphatase) the tyrosine kinase Src act in opposition to each other to regulate NMDAR function. Src enhances NMDAR single-channel gating, resulting in increased NMDAR-mediated synaptic currents in neurons. Src might phosphorylate NMDAR subunits and/or other proteins in the NMDAR complex. STEP61 activity leads to dephosphorylation of Src substrates, thereby reversing Src-mediated upregulation of NMDAR channel gating and resulting in decreased NMDAR-mediated currents. Glu, glutamate; Gly, glycine (Adapted from Salter and Kalia, 2003).

Chapter 2

Differential calcium-dependent modulation of NMDA currents in CA1 and CA3 hippocampal pyramidal cells

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2.1 Abstract

Neuronal Ca^{2+} influx via *N*-methyl-*D*-aspartic acid receptors (NMDARs) is essential for the development and plasticity of synapses, but also triggers excitotoxic cell death when critical intracellular levels are exceeded. Therefore, finely equilibrated mechanisms are necessary to ensure that NMDAR function is maintained within a homeostatic range. Here we describe a pronounced difference in the modulation of NMDA currents in two closely related hippocampal cell types, the CA1 and the CA3 pyramidal cells (PCs). Manipulations that increase intracellular Ca^{2+} levels strongly depressed NMDA currents in CA3 with only minor effects in CA1 PCs. Furthermore, activation of G_q-coupled metabotropic receptors potentiated NMDA currents in CA1 but depressed them in CA3 PCs. Interestingly, the CA3 type modulation of NMDARs could be converted into CA1-like behavior, and vice versa, by increasing Ca^{2+} buffering in CA3 cells or decreasing Ca^{2+} buffering in CA1 cells, respectively. Our data suggest that a differential Ca^{2+} sensitivity of the regulatory cascades targeting NMDARs plays a key role in determining the direction and the magnitude of NMDA responses in various types of neurons. These findings may have important implications for NMDA receptor-dependent synaptic plasticity and the differential sensitivity of CA1 and CA3 PCs to NMDAR-dependent ischemic cell death.

2.2 Introduction

NMDARs are glutamatergic ionotropic receptors that act as gatekeepers for the influx of dendritic Ca^{2+} during synaptic activity. Reflecting the importance of Ca^{2+} entry via NMDARs, several regulatory pathways exist that meticulously control NMDAR activity. Potentiation of NMDARs can be achieved by activating one of several transduction cascades that converge on Src kinase resulting in tyrosine phosphorylation of the receptor (Kotecha and MacDonald, 2003). This mechanism, which is triggered by the activation of metabotropic receptors, increases the open probability without changing the unitary conductance of NMDAR channels (Yu et al., 1997b; Ali and Salter, 2001). Depression of NMDARs occurs through a negative feedback loop that promotes desensitization and inactivation of NMDARs as intracellular Ca^{2+} rises (Kotecha and MacDonald, 2003). Because the activation of metabotropic receptors induces both Src activation and release of Ca^{2+} from intracellular stores, it is difficult to predict whether the final outcome on NMDARs will be up- or down-regulation. In fact, depending on the cell type and experimental conditions, either potentiation or depression of NMDA currents have been reported following activation of metabotropic receptors (Benquet et al., 2002; Kotecha and MacDonald, 2003).

In this study, we investigated the interactions between intracellular transduction pathways mediating positive and negative regulation of NMDARs. We found marked differences in the Ca^{2+}-sensitive metabotropic modulation of NMDARs between CA1 and CA3 PCs.

2.3 Materials and methods

2.3.1 Slice culture preparation and electrophysiology

Hippocampal organotypic slices were prepared from P6 Wistar rats using the roller-tube technique (Gahwiler et al., 1998). After 2–4 weeks in vitro, slice cultures were transferred to a 1 ml recording chamber continuously perfused with Tyrode's solution (1.5ml/min, 30°C) containing: (in mM) 137 NaCl, 2.7 KCl, 11.6 $NaHCO_3$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.6 D-glucose, (in μM) 5 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), 100 picrotoxin, 0.1 tetrodotoxin, 0.001% phenol red, pH 7.4, ~305 mOsm. Whole-cell voltage-clamp recordings were obtained from visualized CA1 and CA3 PCs at –50 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes (2-5 MΩ; series resistance 3-15 MΩ) were filled with: (in mM) 140 K-gluconate, 10 HEPES, 1 EGTA, 4 Mg-ATP, 0.4 Na-GTP, pH 7.2, ~290 mOsm. Liquid junction potentials (-13 mV) were corrected for. When the Ca^{2+} buffer concentration was increased, K-gluconate was reduced to maintain osmolarity. (S)-3,5-dihydroxyphenylglycine (DHPG) experiments were performed with 3 mM extracellular Ca^{2+}. Currents were filtered at 1-2 kHz, stored and analyzed off-line (pClamp7; Axon Instruments). Intracellular recordings were made using an Axoclamp-2A amplifier (Axon Instruments) was used and 40-60 MΩ microelectrodes filled with 1 M potassium acetate. Because of the poor voltage-clamp in cells recorded with microelectrodes, NMDA responses were measured in current-clamp mode.

2.3.2 NMDA current induction

100 μM NMDA was pressure ejected (1 bar) for ~200 ms from a pipette positioned ~100 μm from the soma of recorded cells. NMDA-induced currents were completely blocked by 40 μM (E)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid (CPP). Peak effects of metabotropic agonists (measured after 1 to 5 min) were compared with and normalized to the average NMDA current amplitude from three to five NMDA puffs immediately preceding agonist application (referred to as "baseline"). When testing the effects of different extracellular Ca^{2+} concentrations, the $MgCl_2$ concentration was 2 mM. Solutions were changed after NMDA currents reached a steady state for a given test solution.

2.3.3 Ca^{2+} imaging

20 μM Oregon Green 488 BAPTA-2 was added to the intracellular solution (Kd ~ 580 nM) and excited at 488 nm using a TILL Photonics Polychrome I monochromator (Planegg, Germany). Emitted images were collected with a cooled CCD camera (Princeton Instruments, Trenton, NJ, USA) after passing through a TILL FITC filter set, stored and analyzed using the Axon Imaging Workbench program (Axon Instruments). Images were collected at 10 s intervals (exposure time 200-500 ms). Average fluorescence was determined for regions of interest over the soma (avoiding the nucleus) and the average background fluorescence of a region away from the filled cell was subtracted. ΔF/F was calculated for each image ((average fluorescence - average baseline fluorescence)/average baseline fluorescence).

2.3.4 Statistic

Data are presented as means ± SEM. Potentiation or depression of NMDA currents is given as percentage of the baseline response. Paired Student's *t*-tests were used to compare the non-normalized NMDA currents prior to and following agonist application. To compare effects of changing Ca^{2+} concentration in CA1 and CA3 cells, two-way ANOVAs followed by Tukey's honestly significant difference (HSD) post-hoc tests were used with normalized data (SPSS, SPSS Chicago IL). Values of $p < 0.05$ were considered statistically significant.

2.3.5 Drugs

CPP was kindly provided by Novartis (Basel, Switzerland). Tetrodotoxin was purchased from Latoxan (Valence, France), NBQX from AG Scientific (San Diego, CA), DHPG from Tocris Cookson (Avonmouth, UK), Oregon-green BAPTA-2 and K_4BAPTA were from Molecular Probes (Leiden, Netherlands). Other chemicals were from Sigma. Stock solutions of NBQX and picrotoxin were prepared in DMSO, which never exceeded a final concentration of 0.02%.

2.4 Results

2.4.1 Differential regulation of NMDA responses in CA1 and CA3 PCs by Ca^{2+}

We compared the effect of changing extracellular Ca^{2+} concentration on NMDA responses in CA1 and CA3 PCs. Pharmacologically isolated NMDA currents were induced in PCs voltage-clamped at –50 mV by pressure application of NMDA at 30 sec intervals. Increasing the extracellular Ca^{2+} concentration affected NMDA currents differently in CA1 and CA3 PCs ($p < 0.001$, two-way ANOVA)(Fig. 2.1). 10 mM Ca^{2+} strongly depressed NMDA currents in CA3 cells (41.3 ± 4.2% of response in 0.1 mM Ca^{2+}, $n = 9$, $p < 0.001$, Tukey's HSD), but had much less effect in CA1 cells (80.2 ± 2.4% of response in 0.1 mM Ca^{2+}, $n = 8$, $p < 0.001$, Tukey's HSD) (Fig.2.1).

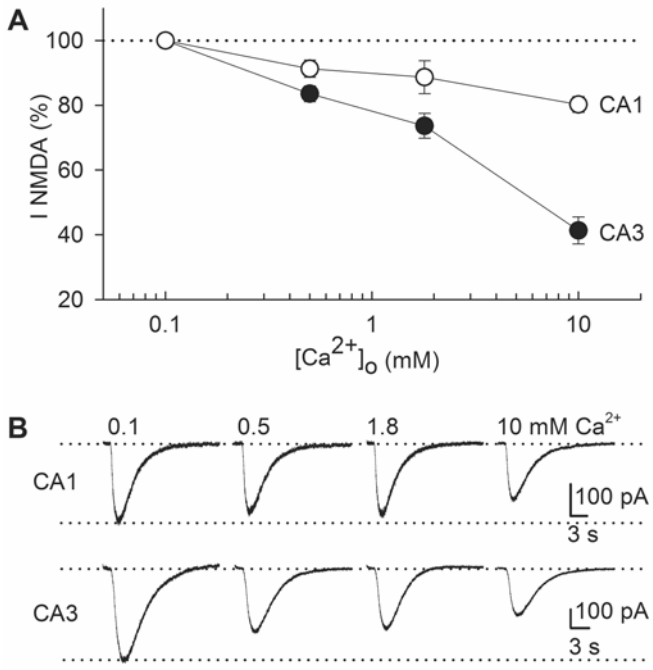


Figure 2.1. **Increasing extracellular Ca^{2+} reduces NMDA currents more strongly in CA3 than in CA1 PCs.** *A*, Normalized NMDA current versus extracellular Ca^{2+} concentration. Note the much steeper concentration-response curve for CA3 cells. *B*, NMDA currents in different Ca^{2+} concentrations from a CA1 and a CA3 PC.

Increasing the extracellular Ca^{2+} concentration is expected to cause a corresponding increase in intracellular Ca^{2+} concentration because of enhanced Ca^{2+} influx through the repetitively activated NMDARs and because of a Ca^{2+} conductance active at a holding potential of –50 mV (Gee et al., 2003). This was confirmed by imaging intracellular Ca^{2+} with Oregon Green 488 BAPTA-2, while simultaneously recording whole cell currents. We wished to compare both the basal Ca^{2+} rises due to changing extracellular Ca^{2+} and the transient Ca^{2+} rises due to activation of NMDARs in

CA3 versus CA1 PCs. To determine the baseline Ca^{2+}, ΔF/F was calculated from the background-subtracted images taking the value at 0.1 mM Ca^{2+} as the baseline. To calculate the Ca^{2+} transients associated with NMDAR activation, the Ca^{2+} level prior to each transient was taken as the baseline. As extracellular Ca^{2+} increased, intracellular Ca^{2+} increased in both CA1 and CA3 PCs ($p < 0.05$, $n = 3$ CA1, $n = 3$ CA3, two-way ANOVA)(Fig. 2.2*A,B,C*), but there was no difference between CA1 and CA3 PCs ($p > 0.10$). Likewise, the Ca^{2+} transients evoked by NMDA increased in both CA1 and CA3 PCs reflecting the increased driving force for Ca^{2+} entry through the NMDA channels ($p < 0.003$, $n = 3$ CA1, $n = 3$ CA3, two-way ANOVA). Again, there was no difference between CA1 and CA3 PCs ($p > 0.5$, two-way ANOVA)(Fig. 2.2*A,B,D,E*). In contrast, the simultaneously recorded NMDA currents were significantly more depressed in CA3 than in CA1 PCs ($p < 0.5$, $n = 3$ two-way ANOVA)(see also Fig. 2.1*A*). Thus, similar manipulation of intracellular Ca^{2+} levels results in markedly different Ca^{2+}-dependent depression of NMDA currents in CA1 and CA3 PCs.

2.4.2 NMDA currents are potentiated in CA1 and depressed in CA3 PCs by metabotropic receptors

As the above experiments show that the increasing intracellular Ca^{2+} depresses NMDA currents, we asked whether similar modulation occurs when intracellular Ca^{2+} levels are raised by activating metabotropic G_q-coupled receptors. Activating metabotropic receptors enhances NMDA currents in hippocampal PCs via a second messenger cascade involving phosphoinositol hydrolysis and subsequent phosphorylation of NMDA channels via the protein tyrosine kinase Src (Kotecha and MacDonald, 2003). As expected, we observed that application of the cholinergic agonist muscarine (10 μM for 2 min) potentiated NMDA currents in CA1 PCs (140.6 ± 2.79%, $n = 5$, $p < 0.01$) (Fig. 2.3*A*). In contrast, muscarine induced a transient potentiation followed by a strong depression of NMDA currents in CA3 PCs obtained from the same slice cultures (68.2 ± 1.6%, $n = 5$, $p < 0.01$) (Fig. 2.3*A*). The potentiation of NMDA currents in CA1 and the depression in CA3 were reversible after approximately 15 minutes of muscarine washout.

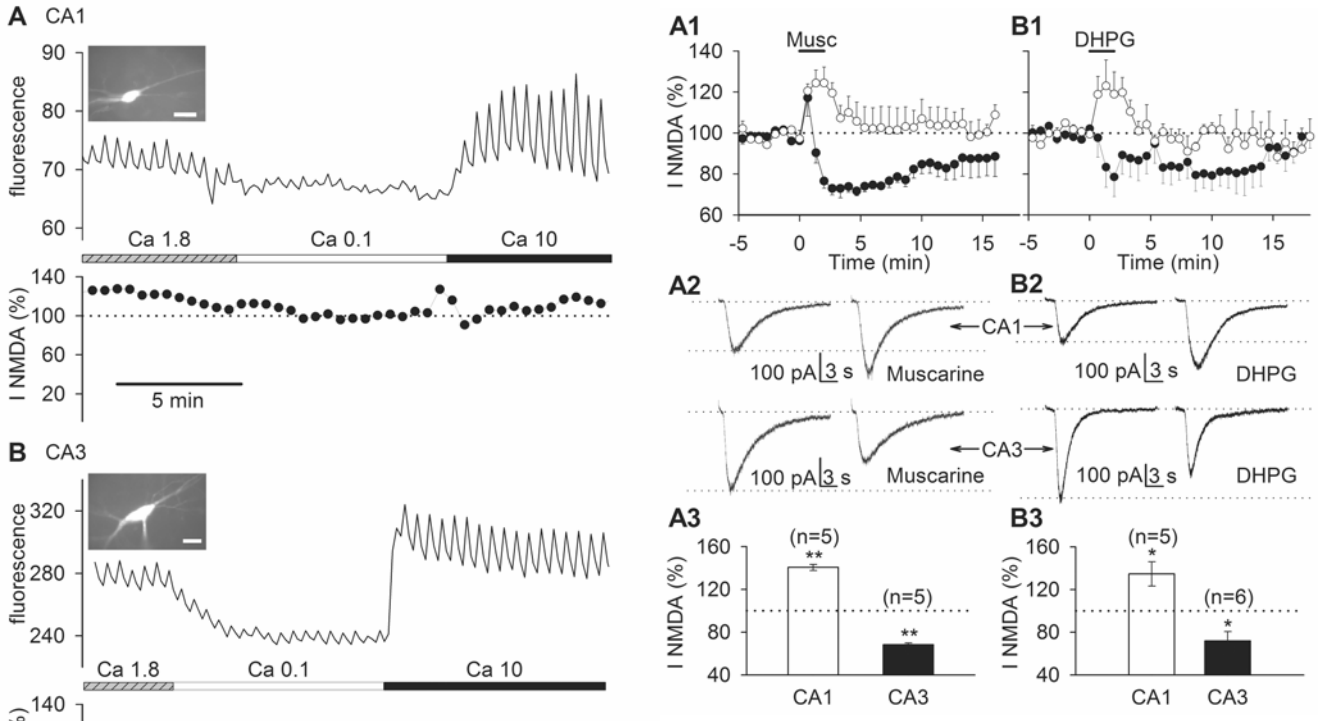


Figure 2.3. **Activating muscarinic or metabotropic glutamate receptors potentiates NMDA currents in CA1 and depresses NMDA currents in CA3 PCs.** *A1,* Normalized NMDA currents in CA1 (○) and in CA3 (●) PCs from 5 slices before and after application of muscarine (Musc, 10 μM). *B1,* Effects of DHPG (50 μM) on normalized NMDA currents. NMDA currents before and after application of muscarine (*A2*) or DHPG (*B2*). Pooled maximal effects of muscarine (*A3*) or DHPG (*B3*)(*p < 0.05, **p < 0.01).

Figure 2.2. **Increasing extracellular Ca^{2+} concentration causes similar increases in intracellular Ca^{2+} levels and NMDA-induced Ca^{2+} transients in CA1 and CA3 PCs, whereas simultaneously recorded NMDA currents are strongly depressed only in CA3.** A CA1 *(A)* and a CA3 *(B)* PC were filled with the Ca^{2+} indicator Oregon Green 488 BAPTA-2 and fluorescence images were collected every 10 s. Top traces show fluorescence signals (background subtracted arbitrary units) with the simultaneously recorded normalized NMDA-induced currents below. Note the transient rises in the fluorescence signal corresponding to Ca^{2+} influx through the NMDARs. Extracellular Ca^{2+} was changed where indicated. CPP completely blocked responses to NMDA. *C,* The increase in ΔF/F relative to the basal level in 0.1 mM was the same in CA1 (○) and CA3 (●) PCs when extracellular Ca^{2+} was increased. *D,* The change in fluorescence induced by NMDA application increased with increasing Ca^{2+} concentration in both CA1 and CA3 PCs. *E,* Sample Ca^{2+} transients and NMDA currents from the cells in *A,B.* Note that NMDA currents are much more sensitive to changes in Ca^{2+} concentration in CA3 than in CA1 cells. Scale bars: *A,B,* 20 μm (*A*), *E*, Ca^{2+} transients, 2 min, CA1 0.5 ΔF/F, CA3 0.1 ΔF/F, NMDA currents, 3 s, 100 pA.

The difference in NMDA receptor modulation in CA1 versus CA3 PCs was also observed with activation of group I metabotropic glutamate receptors. Application of DHPG (50 μM for 2 min), a group I metabotropic glutamate receptor agonist, markedly potentiated NMDA responses in CA1 PCs (134.5 ± 11.4%, $n = 5$, $p < 0.05$) as described previously (Valenti et al., 2002; Kotecha and MacDonald, 2003), whereas NMDA responses were depressed in CA3 PCs (71.8 ± 8.9%, $n = 6$, $p < 0.05$) (Fig. 2.3*B*). These results indicate that increases in intracellular Ca^{2+} levels have opposite effects on the metabotropic transduction mechanisms modulating NMDARs in CA3 versus CA1 PCs. This idea was tested further by examining the effects of changing intracellular Ca^{2+} buffering on the modulation of NMDA currents in these two types of PCs.

2.4.3 Intracellular Ca^{2+} buffering alters metabotropic modulation of NMDA currents

In CA1 PCs, when Ca^{2+} buffering was decreased by lowering intracellular EGTA from 1 mM to 0.1 mM, muscarine depressed rather than potentiated NMDA currents (83.3 ± 2.7%, $n = 5$, $p < 0.05$) (Fig. 4*A*). Conversely, increasing intracellular Ca^{2+} buffering with 30 mM BAPTA prolonged the muscarinic potentiation of NMDA currents (149.5 ± 11.8%, $n = 5$, $p < 0.05$) (Fig. 2.4*A*). In CA3 PCs, the depression of NMDA currents seen with 1 mM EGTA intracellularly (71.5 ± 2.0%, $n = 9$, $p < 0.001$) was switched to a significant potentiation with 30 mM BAPTA (134.3% ± 8.9, $n = 6$, $p < 0.01$) (Fig. 2.4*B*) mimicking the effect of muscarine on NMDA currents in CA1 PCs loaded with 1 mM EGTA (compare with Fig. 2.3*A*). Intermediate Ca^{2+} buffering with 10 mM EGTA resulted in an intermediate degree of muscarinic modulation of NMDA responses (125.0 ± 17.4%; $n = 4$, $p > 0.10$ in CA3, data not shown). Likewise, the DHPG-induced depression of NMDA currents in CA3 PCs was converted into potentiation when intracellular Ca^{2+} buffering was enhanced with 10 mM BAPTA (142.8 ± 9.6%, $n = 5$, $p < 0.05$) (Fig. 2.4*B*, inset). Thus, the modulation of NMDARs by muscarinic or metabotropic glutamate receptors depends critically on intracellular Ca^{2+}.

We next examined whether the pronounced Ca^{2+}-dependent depression of NMDA currents in response to increased extracellular Ca^{2+} in CA3 PCs is changed by increasing intracellular Ca^{2+} buffering. When 30 mM BAPTA was used as the

intracellular buffer, a virtually flat concentration-response curve reminiscent of that in CA1 cells was observed (Fig. 2.4*C*, 88.0 ± 4.8% of response in 0.1 mM Ca^{2+} at 10 mM Ca^{2+}, $n = 5$, $p < 0.001$ two-way ANOVA; for comparison see Fig. 2.1*A*).

To ensure that the differential Ca^{2+}-dependent modulation of NMDA responses occurs in PCs with unmanipulated Ca^{2+} buffering, we recorded NMDA-induced depolarizations with sharp microelectrodes. Under these conditions, Ca^{2+} buffers are not introduced into the neurons and intracellular dialysis is minimal. Again we found a significant difference in the modulation of NMDA responses between both cell types. In response to 1 μM muscarine, NMDA responses were strongly and irreversibly potentiated in an LTP-like fashion in CA1 (236.8 ± 38.9%, $n = 5$, $p < 0.05$), whereas in CA3 PCs potentiation was significantly less and of short duration (5-6 min, 135.1 ± 5.4%, $n = 5$, $p < 0.01$). The fact that we observe this slight transient potentiation rather than a depression in CA3 PCs when recording with microelectrodes suggests that intrinsic Ca^{2+} buffering in this cell type is greater than that observed with 1 mM EGTA in the patch-clamp experiments and very similar to the modulation obtained with 10 mM EGTA (1mM EGTA: 71.5 ± 2.0%, $n = 9$, $p < 0.001$ versus 10 mM EGTA: 125.0 ± 17.4%; $n = 4$, $p > 0.10$ in CA3, data not shown). These findings confirm that the differential Ca^{2+}–dependent regulation of NMDA responses in CA1 versus CA3 PCs is inherent to the cell type.

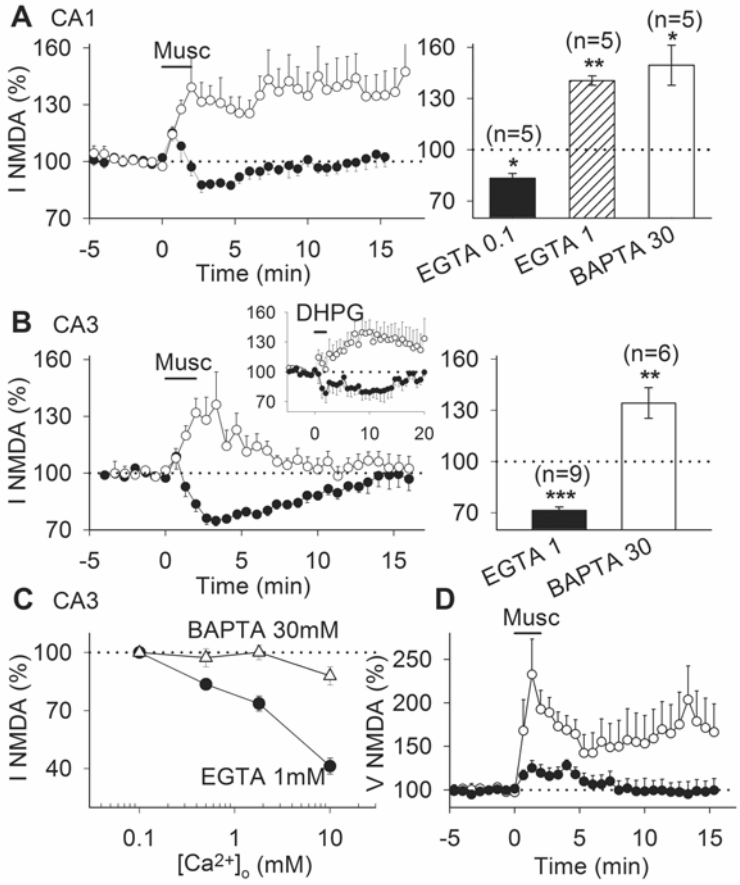


Figure 2.4. **Intracellular Ca^{2+} buffering changes the polarity and the amplitude of metabotropic modulation of NMDA currents.** *A,* Normalized peak NMDA currents in CA1 PCs with low intracellular Ca^{2+} buffering (0.1 mM EGTA ●), and high Ca^{2+} buffering (BAPTA 30 mM ○) with pooled maximal potentiation/depression at right. *B,* Normalized peak NMDA currents in CA3 PCs with 1 mM EGTA ●, and high Ca^{2+} buffering (BAPTA 30 mM ○). *inset,* DHPG (50 μM) effect on NMDA currents in CA3 PCs with 1 mM EGTA (●) or 30 mM BAPTA (○) with pooled maximal potentiation/depression at right. *C,* Effects of changing extracellular Ca^{2+} concentration on NMDA currents in CA3 PCs. (EGTA 1 mM ●, BAPTA 30 mM △). *D,* Normalized NMDA-induced depolarizations recorded intracellularly in CA1 (○) and CA3 (●)PCs (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

2.5 Discussion

Our experiments show that Ca^{2+}-dependent depression of NMDA responses is greater in hippocampal CA3 PCs than in CA1 PCs. This effect was observed by altering intracellular Ca^{2+} levels through changes in extracellular Ca^{2+} concentration or by altering intracellular Ca^{2+} buffers. Strikingly, this Ca^{2+}-dependent mechanism resulted in distinct modulation in the two cell types when activating G_q-coupled metabotropic receptors, such that activation of muscarinic receptors or mGluRs typically depressed NMDA responses in CA3 PCs but potentiated them in CA1 PCs.

It is well established that Ca^{2+} influx through NMDARs triggers a negative feedback process that inactivates NMDA receptors through the activation of various Ca^{2+}-dependent proteins including calmodulin and Ca^{2+}-dependent phosphatases (Kotecha et al., 2003). On the other hand, NMDA responses can be potently upregulated by activation of G_q-coupled metabotropic receptors, via a protein kinase C (PKC)-dependent (Ben Ari et al., 1992; Lu et al., 1999; Kotecha and MacDonald, 2003) or a PKC-independent pathway (Benquet et al., 2002; Heidinger et al., 2002) both of which culminate in Src-mediated tyrosine phosphorylation of NMDARs (Ali and Salter, 2001). Our data demonstrate that both the positive and the negative modulatory pathways are present and converge on NMDARs in CA1 and CA3 PCs, but that a difference in the balance between opposing signaling cascades can result in an opposite net modulatory effect. Both potentiation and depression of NMDA responses following metabotropic glutamate receptor activation have been reported (reviewed in (Benquet et al., 2002; Kotecha and MacDonald, 2003). Likewise, stimulation of muscarinic receptors potentiates NMDARs in CA1 PCs (Markram and Segal, 1990b), whereas we found that depression predominated in CA3 PCs. We propose that cell-type specific differences in intrinsic Ca^{2+} signaling or buffering, and the intracellular Ca^{2+} concentrations set by the experimental conditions determine which modulatory pathway prevails and results in either potentiation or depression of NMDARs by metabotropic receptors.

Although an increase in intracellular Ca^{2+} concentration primarily depresses NMDARs, very strong Ca^{2+} buffering blocks the potentiation (Calabresi et al., 1998; Skeberdis et al., 2001; Kotecha and MacDonald, 2003). The absolute requirement for Ca^{2+} in the potentiating pathways reflects the presence of the Ca^{2+}-dependent proteins PKC or calmodulin, both of which activate the tyrosine kinase Pyk2/CAKβ that, in turn,

induces Src-dependent phosphorylation of NMDARs (Heidinger et al., 2002; Kotecha and MacDonald, 2003). Therefore, metabotropic receptor activation will maximally potentiate NMDARs under physiological conditions when concentrations of intracellular Ca^{2+} are low, and shifts in intracellular Ca^{2+} concentration in either direction will reduce NMDAR potentiation or induce depression (i.e. a bell shaped curve).

The signaling pathways leading to potentiation of NMDARs have been mapped out in considerable detail. In contrast, the processes underlying the Ca^{2+}-dependent inactivation of NMDA receptors are less understood. A number of Ca^{2+}-dependent proteins including calmodulin, calcineurin, PKC and α-actinin-2 are implicated in the regulation of NMDAR function (for discussion, see (Rycroft and Gibb, 2002b). Of particular interest in light of our data is the recent report that striatal-enriched protein tyrosine phosphatase (STEP), which is also expressed in hippocampus, is activated by calcineurin as a result of Ca^{2+} entry through NMDARs (Paul et al., 2003). Thus, the positive and negative signaling pathways modulating NMDARs can target respectively the tyrosine kinase Src and the tyrosine phosphatase STEP and bi-directionally control the phosphorylation state of NMDARs (Pelkey et al., 2002). Low intracellular Ca^{2+} could therefore favor the potentiating Src-dependent pathway, increased intracellular Ca^{2+} could favor NMDAR depression via STEP and a lack of Ca^{2+} would activate neither pathway.

How can the differences in NMDAR modulation in CA1 versus CA3 PCs be explained? Numerous factors could contribute including 1) differential expression of the metabotropic receptors, 2) differential expression of NMDAR subunits 3) the presence of different pathways coupling the metabotropic and NMDA receptors, and 4) differential Ca^{2+} handling. The first possibility is unlikely as experimentally altering Ca^{2+} concentration, thereby bypassing the metabotropic receptors, led to stronger depression of NMDA currents in CA3 than in CA1 cells. With respect to the second possibility, immunohistochemical studies report similar expression patterns of NMDA receptor subunits in CA1 and CA3 PCs in rat hippocampus (Fritschy et al., 1998) including in slice cultures (Gerfin-Moser et al., 1995). However, in CA3 PCs there is marked heterogeneity in NMDA receptor expression at mossy fiber versus associational fiber synapses (Watanabe et al., 1998) and at apical versus basal dendrites (Kawakami et al., 2003). It will be interesting to compare the consequences of metabotropic

modulation of NMDARs at specific synapses in the future. Third, we predict that differences in the Ca^{2+} sensitivity of the signaling cascades coupling metabotropic receptors and NMDARs contribute to the differential responses recorded in CA1 and CA3 PCs. Fourth, our experiments provide direct evidence that differential Ca^{2+} handling is critical in determining the direction of NMDAR modulation in the two cell types. By manipulating intracellular Ca^{2+} buffering we were able, with respect to NMDAR modulation, to convert a CA3 into a CA1 PCs, and vice-versa. This finding suggests that there may be a substantially higher Ca^{2+} buffering capacity in CA1 PCs. An attractive candidate for endogenous Ca^{2+} buffering is calbindin-D_{28K}, which is expressed in CA1 but not in CA3 PCs (Sloviter, 1989). Furthermore, LTP in CA1 PCs is impaired by a calbindin deficiency, but is rescued by chelating intracellular Ca^{2+} (Jouvenceau et al., 2002). Our observation of a similar rise in free intracellular Ca^{2+} in CA1 and CA3 PCs in response to raising extracellular Ca^{2+} appears to speak against a difference in endogenous buffering capacity. However, critical signaling events may occur within functional microdomains with localized differences in Ca^{2+} concentration that cannot be resolved against the bulk changes in cytoplasmic Ca^{2+} levels or that are inaccessible to the Ca^{2+} indicators.

Our findings have a number of functional implications. Depending on the intracellular Ca^{2+} availability, metabotropic receptors can potentiate or depress NMDARs and can therefore act as a switch to determine whether synapses undergo long-term potentiation or long-term depression (Bortolotto et al., 1999). This mechanism may explain why the threshold for NMDA receptor-dependent LTP induction is lower at the Schaffer-collateral - CA1 synapse than at the associational fiber – CA3 synapse (Moody et al., 1998). Furthermore, the propensity for NMDARs to become potentiated in CA1 PCs may explain, in part, the selective vulnerability of these neurons to ischemic cell death (Schmidt-Kastner and Freund, 1991) whereas in CA3 PCs the excessive Ca^{2+} influx during an excitotoxic insult may be neuroprotective by shutting down NMDARs.

Chapter 3

Muscarinic receptor stimulation reduces NMDA responses in CA3 hippocampal pyramidal cells via Ca^{2+}-dependent activation of tyrosine phosphatase

Anton A. Grishin, Pascal Benquet, Urs Gerber

(Neuropharmacology, in press)

3.1 Abstract

NMDA-type glutamate receptors perform critical functions during the development of the nervous system and in the initiation of synaptic plasticity. An important mechanism in setting the gain of NMDA receptors involves the stimulation of G-protein-coupled receptors (GPCRs), which through activation of protein tyrosine kinases leads to an upregulation of NMDA receptors. In contrast, little is known about how NMDA receptors are downregulated. In the present study, we characterized a signaling pathway that mediates the depression of NMDA receptor function in response to stimulation of muscarinic acetylcholine receptors. Whole-cell patch-clamp recordings obtained from CA3 pyramidal cells in organotypic slice cultures revealed that under conditions of low intracellular calcium buffering application of muscarine depressed NMDA receptor current. The sensitivity of this response to pirenzipine indicated that the M1 acetylcholine receptor is mediating this depression. The muscarine-induced depression of NMDA current was prevented by blocking G-protein function or after depleting intracellular Ca^{2+} stores with cyclopiazonic acid. Inhibitors of calmodulin prevented the depression whereas blocking calcineurin enhanced the depression of NMDA currents. Blocking tyrosine phosphatase activity with pervanandate converted the muscarine-induced depression into a potentiation of NMDA currents, whereas blocking protein kinase A (H-89), Src kinase (PP2, SU6656), or PKC (GF 109203X) failed to prevent the depression of NMDA currents.

As Src tyrosine kinase is known to phosphorylate and upregulate NMDA receptors, we propose that a protein tyrosine phosphatase(s) counteracting the action of Src is the final target in the mAChR-dependent inhibitory signaling cascade. Our data are consistent with a transduction cascade comprising an M1 acetylcholine receptor → G-protein → Ca^{2+} release → calmodulin → tyrosine phosphatase.

3.2 Introduction

N-methyl-D-aspartate (NMDA) receptors are ionotropic glutamate receptors critical for synaptic plasticity, which is thought to underlie learning and memory. Over-activation or defective regulation of NMDA receptors (NMDAR) are, however, implicated in diverse brain disorders. Numerous transduction pathways converge onto the NMDA receptors, thereby setting their gain and ultimately the efficacy of synaptic transmission (Kotecha and MacDonald, 2003). A well-characterized form of NMDA receptor modulation is initiated by the activation of G_q protein-coupled metabotropic receptors. Various types of metabotropic receptors are expressed in close proximity to synaptic NMDA receptors and, in the case of the metabotropic glutamate receptors, have been shown to be physically linked with them via a complex of scaffolding and adaptor proteins (Sheng and Kim, 2002). Previous studies have focused mainly on the mechanisms through which neurotransmitters acting at metabotropic receptors upregulate NMDA receptor function (Kotecha and MacDonald, 2003). A common motif in this type of potentiation of NMDA responses is the activation of signaling cascades that converge on non-receptor tyrosine kinases belonging to the Src family kinases (SFKs), which then leads to the phosphorylation of the NR2A or NR2B subunits or proteins coupled to the NMDA receptor complex to increase channel gating (Salter and Kalia, 2004). Less is currently known about the processes underlying the decrease in NMDA receptor function in response to neurotransmitter activation of G_q protein-coupled metabotropic receptors. In analogy to better characterized biochemical systems accruing evidence indicates that SFK-mediated phosphorylation and upregulation of NMDA receptors is counterbalanced through the activation of protein tyrosine phosphatases that dephosphorylate NMDA receptors, thus allowing the bidirectional control of NMDA receptor function (Salter and Kalia, 2004). Indeed, various protein tyrosine phosphatases are associated with the NMDA receptor complex and the pharmacological blockade of protein tyrosine phosphatases in excised membrane patches increases NMDA receptor gating (Wang et al., 1996). However, the upstream signaling events leading to the activation of protein tyrosine phosphatases in neurons are poorly characterized and whether GPCRs can trigger these signaling cascades is currently unknown.

In a previous study we have shown that, surprisingly, in hippocampal pyramidal cells the same G_q-coupled GPCRs (muscarinic acetylcholine receptor (mAChRs) and

metabotropic glutamate receptor (mGluRs)) are simultaneously coupled to a stimulatory and an inhibitory pathway modulating NMDAR function in a Ca^{2+}-dependent and cell type specific manner (Grishin et al., 2004). In the present study we examined the signaling cascade of the inhibitory pathway leading to the depression of NMDA currents upon stimulation of mAChRs in CA3 pyramidal neurons. Here we show that stimulation of M1 muscarinic receptor leads to Ca^{2+}/calmodulin-dependent activation of protein tyrosine phosphatase(s), which results in the depression of NMDA receptor function.

3.3 Methods

3.3.1 Hippocampal Slice Culture Preparation

Hippocampal organotypic slices were prepared from P6 Wistar rats using the roller-tube technique (Gahwiler et al., 1998). After 2–4 weeks in vitro, slice cultures were transferred to a 1 ml recording chamber continuously perfused with a saline solution (1.5ml/min, 30°C) containing: (in mM) 137 NaCl, 2.7 KCl, 11.6 $NaHCO_3$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.6 D-glucose, (in μM) 0.5 tetrodotoxin, 5 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), 100 picrotoxin, 0.001% phenol red, pH 7.4, ~305 mOsm.

3.3.2 Whole-Cell Recordings

Somatic whole-cell voltage-clamp recordings were obtained from visualized CA3 pyramidal neurons held at –50 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes with a resistance of 2-5 MΩ were pulled from glass capillaries (o.d. 1.5 mm, i.d. 1.16 mm; Harvard Apparatus Ltd, UK) using a vertical two-stage puller L/M 3P-A (List Electronics, Darmstadt, Germany). The standard intracellular solution contained (in mM): 140 K-gluconate, 10 HEPES, 1 EGTA, 4 Mg-ATP, 0.4 Na-GTP, pH ~7.2, ~290 mOsm. Liquid junction potentials (-13 mV) were corrected for. Series resistance (3-15 MΩ) and input resistance were monitored regularly. Electronic compensation of series resistance was not employed. Currents were filtered at 1kHz, stored and analyzed off-line. Data were acquired, stored and analyzed using pClamp software (pClamp7; Axon Instruments).

3.3.3 NMDA Current Induction

200 μM NMDA was pressure ejected (1 bar) for ~150 ms from a pipette positioned ~100 μm from the soma of the recorded cell. NMDA-induced currents were completely blocked by 40 μM (E)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid (CPP). NMDA current amplitudes were measured from the baseline holding current to the peak. The peak effect of muscarine was compared with and normalized to the average NMDA current amplitude from three to six NMDA applications immediately preceding application of the agonist (referred to as “baseline”). I-V curves were determined with a ramp protocol from -70 to 0 mV (2 sec duration) run before and during NMDA pressure application, and the respective traces were subtracted to obtain

the I–V curve of the NMDA response. This procedure was repeated for control conditions and after muscarine.

3.3.4 Statistics Analysis

Data are presented as means ± SEM. Paired Student's *t*-tests were used to compare the non-normalized NMDA currents prior to and following muscarine application. The unpaired t-test was used to compare the effects of two treatments on normalized NMDA currents in different neurons. Values of $p < 0.05$ were considered statistically significant.

3.3.5 Chemicals

CPP was kindly provided by Novartis (Basel, Switzerland), tetrodotoxin was purchased from Latoxan (Valence, France), NBQX from AG Scientific (San Diego, CA), pirenzipine from Biberach/Riss (Switzerland), QX-314 from Alamone labs (Jerusalem, Israel), cyclopiazonic acid and GF 109203X from Tocris, FK-506 Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), PP2 from Calbiochem, SU6656 from Biaffin (Kassel, Germany), sodium orthovanadate from Alexis Corporation (Läufelfingen, Switzerland). NMDA, AMPA, picrotoxin, muscarine, GDPβS, W-13, calmidazolium, rapamycin, nifedipine, H-89 were from Sigma. Stock solutions of NBQX, picrotoxin, CPA, calmidazolium, FK-506, rapamycin, nifedipine, H-89, GF 109203X, PP2, and SU6656 were prepared in DMSO, which never exceeded a final concentration of 0.02%. Pervanadate was prepared by heating freshly dissolved 100 mM Na orthovanadate in H_2O to 95 °C then cooling to 25 °C on ice and adjusting pH to 10. Just before use 1 part 500 mM H_2O_2 was combined with 50 parts of the 100 mM Na orthovanadate, left to stand for 10 minutes, and then diluted 1:1000 in saline and applied. Pharmacological agents were introduced into the bath 15-20 min before co-application of muscarine as indicated by the black bars in the figures. In experiments with SU6656, we had to preincubate the cultures with the antagonist for 2 hours to block Src.

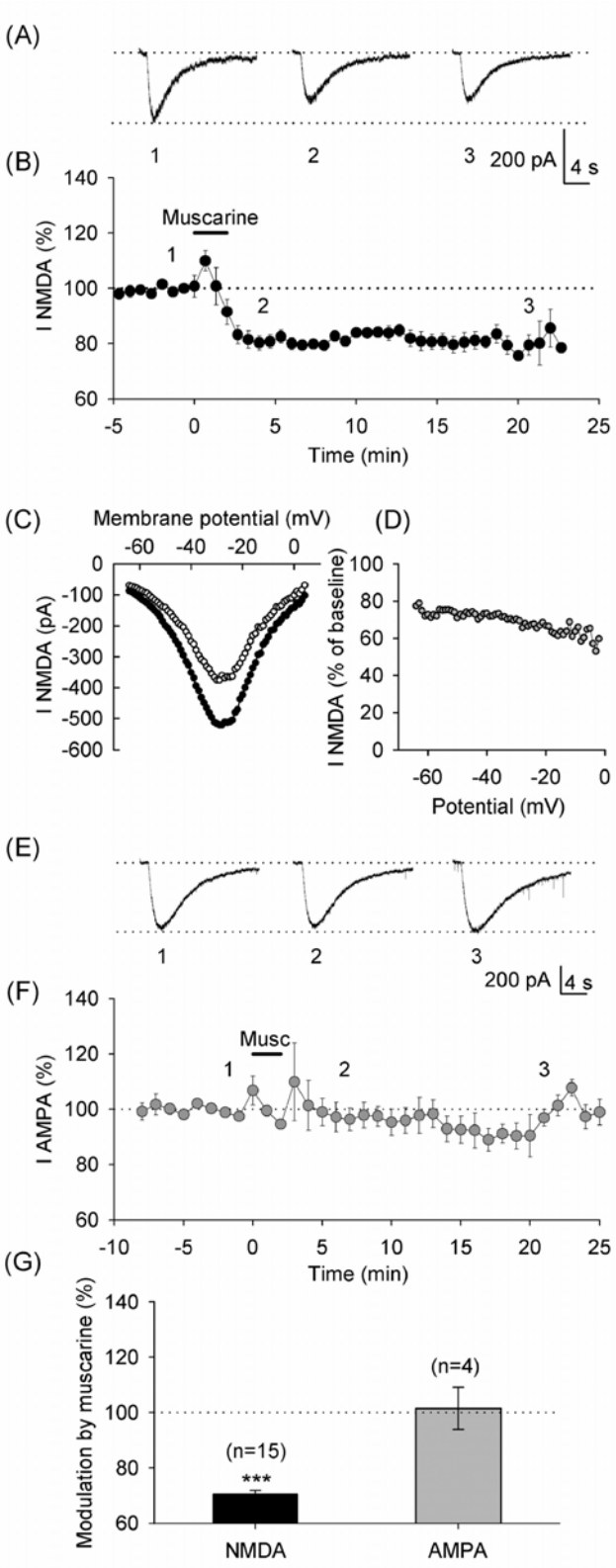
3.4 Results

3.4.1 Activation of Muscarinic Cholinergic Receptors Depresses NMDA Currents in CA3 Hippocampal Pyramidal Cells

We have previously shown that under conditions of strong intracellular calcium buffering (30 mM BAPTA) activation of muscarinic cholinergic receptors (mAChRs)(Grishin et al., 2004) enhances responses mediated by NMDA-type glutamate receptors in CA3 pyramidal cells. On the other hand, when intracellular calcium is weakly buffered (1 mM EGTA), bath-application of muscarine (10 μM for 2 min) induced a long-lasting depression of NMDA receptor-mediated currents induced by brief pressure application of NMDA to a CA3 pyramidal cell voltage-clamped at –50 mV (Fig. 3.1A,B,G, (Grishin et al., 2004)). Peak NMDA currents were inhibited to 70.5 ± 1.3% of baseline (n = 15, p < 0.001) for at least 30 min except in 4 cells where inhibition recovered within 15 min. The magnitude of the muscarine-induced depression of NMDA current was independent of membrane potential over the range from -70 to 0 mV, Fig. 1C,D). This negative modulation by muscarine was specific for the NMDA-type of ionotropic glutamate receptor. The same application of muscarine (10 μM for 2 min) had no effect on currents mediated by AMPA-type ionotropic glutamate receptors. Responses to brief pressure application of AMPA remained at 101.5 ± 7.6% of baseline in the presence of muscarine (n = 4, p > 0.5) (Fig. 3.1E,F,G).

3.4.2 Muscarine-Induced Depression of NMDA Currents Requires Activation of M1 Muscarinic Receptors, G-proteins and Subsequent Ca^{2+} Release

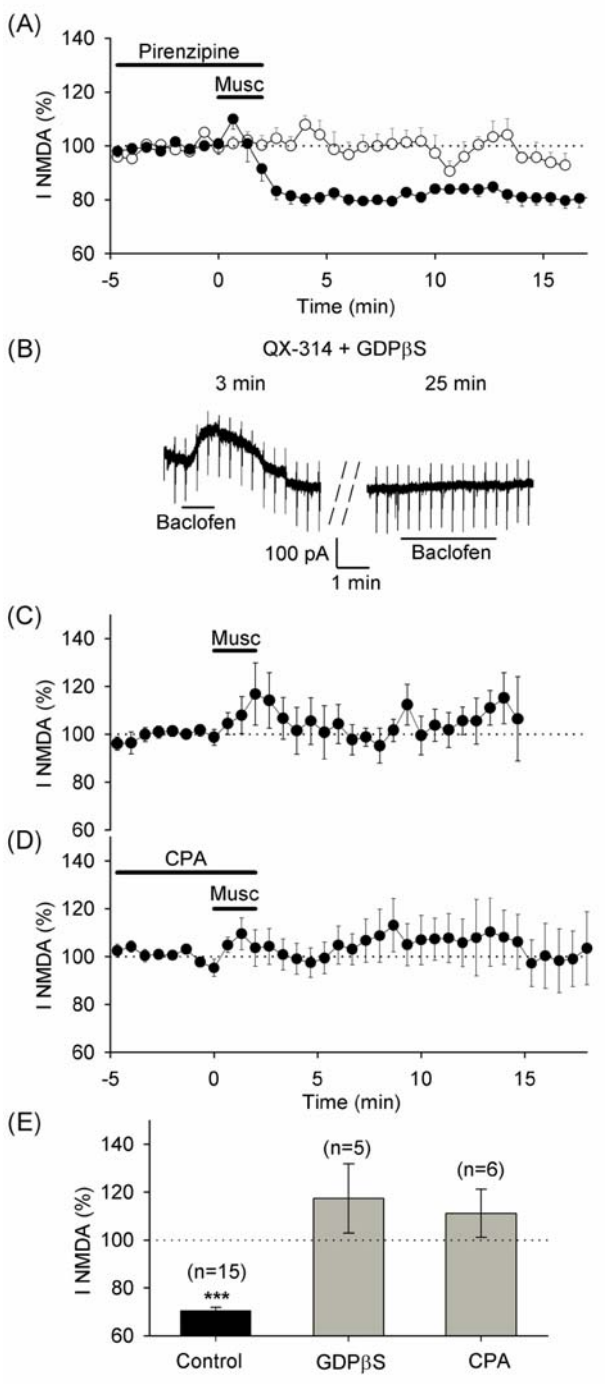
Modulation of NMDA receptors by G_q-coupled receptors is Ca^{2+}-dependent (Benquet et al., 2002; Grishin et al., 2004), suggesting the involvement of a Ca^{2+} release pathway in the depression of NMDA currents. Three of the five subtypes of mAChRs, M1, M3, and M5, are coupled to a G_q–dependent pathway that stimulates phospholipase C (PLC) resulting in IP_3–induced Ca^{2+} release. As the M1-type mAChR is most abundant in the hippocampus (Levey et al., 1991) and has been shown to mediate the potentiation of NMDA responses (Marino et al., 1998), we used pirenzipine, a relatively selective M1 antagonist, to determine whether this receptor subtype mediates the inhibitory effect of muscarine on NMDA current. We found that pirenzipine (1 μM, 15



min preincubation) completely blocked the effects of muscarine on NMDA responses (101.7 ± 3.7% of baseline, n = 6, p > 0.1) (Fig. 3.2A). As muscarine can

trigger both G-protein-dependent as well as G-protein-independent signaling (Heuss and Gerber, 2000), we tested whether the depression of NMDA responses by muscarine requires the activation of G-proteins. G-protein function was blocked by dialyzing cells with an intracellular solution containing GDPβS (1 mM) (Eckstein et al., 1979) to which QX-314 (5 mM) was added for a more rapid effect (Hollmann et al., 2001) and blockade was confirmed by demonstrating the complete inhibition of the βγ subunit-activated GIRK current induced by baclofen (20 μM, 1-5 min), a $GABA_B$ agonist (n = 5; Fig. 3.2B).

Figure 3.1. **Muscarine depresses NMDA but not AMPA mediated currents in CA3 hippocampal pyramidal cells.** (A) Representative traces of NMDA-induced whole-cell currents in CA3 hippocampal pyramidal cells before and after application of muscarine. (B) Averaged time course of muscarine-induced depression of NMDA currents. (C) A plot of the subtracted I-V relationship of NMDA currents recorded before (filled circles) and during (open circles) muscarine action. (D) The muscarine-induced depression as a function of membrane potential. (E) Representative traces of whole-cell AMPA-mediated currents recorded before and after application of muscarine. (F) Lack of effect of muscarine on AMPA responses. (G) Pooled maximal effect of muscarine on the amplitudes of NMDA and AMPA currents.



In these cells, the subsequent application of muscarine failed to depress NMDA currents (117.4 ± 14.5% of baseline n = 5, p > 0.05) (Fig. 3.2C,E). Next we tested whether the G-protein-dependent depression of NMDA currents involved intracellular release of Ca^{2+}. Intracellular Ca^{2+} stores were depleted by bath-application of cyclopiazonic acid (20 μM, 20 min), an antagonist of sarco/endoplasmic reticulum calcium ATPase (SERCA) that prevents refilling of the endoplasmic reticulum with Ca^{2+}, followed by brief depolarization to 0 mV to ensure emptying of Ca^{2+} stores. Co-application of muscarine now failed to depress NMDA currents (111.1 ± 10% of baseline, n = 6, p > 0.05) (Fig. 3.2D,E). These results indicate that muscarine-induced depression of NMDA currents in CA3 hippocampal neurons is mediated via activation of M1 muscarinic receptors, which leads to G-protein-dependent Ca^{2+} release from intracellular stores.

Figure 3.2. **The depression of NMDA currents by muscarine involves activation of M1 receptors and G-protein-mediated Ca^{2+} release.** (A) Effect of pirenzipine on the averaged time course of muscarine-induced depression of NMDA currents (open circles– pirenzipine, filled circles – control curve). (B) Dialysis with 5 mM QX-314 and 1mM GDPβS completely blocks G-protein mediated baclofen-induced outward current. (C) G-protein blockade abolishes the muscarine-induced depression of NMDA currents. (D) Pretreatment with the SERCA pump antagonist cyclopiazonic acid abolishes the muscarine-induced depression. (E) Pooled data showing effects of muscarine on NMDA currents after G-protein block and preincubation with cyclopiazonic acid.

3.4.3 The Depression of NMDA Current in Response to Intracellular Release of Ca^{2+} Requires the Activation of Calmodulin

A prime intracellular target of Ca^{2+} is calmodulin, a molecule highly expressed in hippocampal neurons (Gnegy, 2000). To determine whether calmodulin is involved in the Ca^{2+}–dependent depression of NMDA currents by muscarine, two specific calmodulin antagonists were tested. Both W-13 (20 μM, 15-20 min) and calmidazolium (20 μM, 15-20 min) fully blocked muscarinic depression of NMDA current. Application of muscarine did not significantly increase NMDA current (112.6 ± 15.8% of baseline, $n = 5$, $p > 0.05$ and 127.5 ± 11.4%, $n = 5$, $p > 0.05$) after incubation with W-13 and calmidazolium, respectively (Fig. 3.3A,B,C).

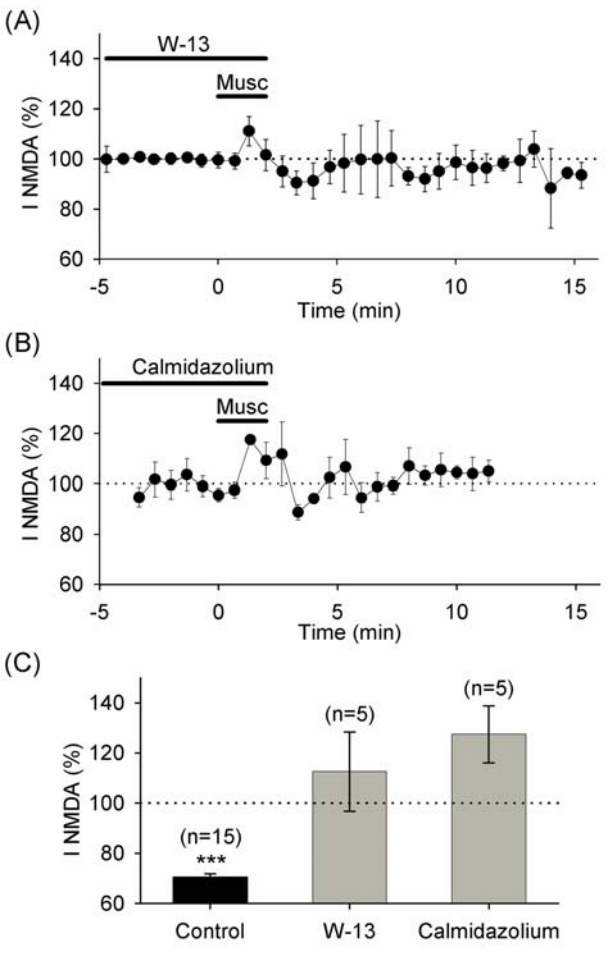


Figure 3.3. **Calmodulin antagonists block muscarine-induced depression of NMDA currents**. Preincubation with W-13 (*A*) or calmidazolium (*B*) abrogates the muscarine-induced depression. (C) Pooled data showing averaged maximal effect of muscarine on NMDA currents after pretreatment with calmodulin antagonists.

3.4.4 Calcineurin Blockade Enhances the Muscarine-Induced Depression of NMDA Currents

An increase in intracellular Ca^{2+} leads to a rapid Ca^{2+}/calmodulin-dependent activation of calcineurin (or PP2B), a protein serine/threonine phosphatase with a high affinity and low dissociation constant for Ca^{2+} (Winder and Sweatt, 2001). Furthermore, calcineurin colocalizes with NMDA receptors and reduces unitary NMDA currents (Lieberman and Mody, 1994). These properties suggest a role for calcineurin in the signaling mechanism mediating the depression of NMDA currents by muscarine. We tested for an involvement of calcineurin in the depression of NMDA currents by muscarine using the selective antagonist FK-506 and the inactive analog rapamycin. Surprisingly, however, after blocking calcineurin with the selective antagonist FK-506 (10 μM, 20 min) application of muscarine induced an even greater depression of NMDA currents (52.6 ± 5.9% of baseline, n = 5, p < 0.001) (Fig. 4A,B). Pretreatment with the inactive analog rapamycin (10 μM) had no significant effect on the muscarinic depression of NMDA currents (70.2 ± 2.6% n = 4, versus control 70.5 ± 1.3% n =15; $p > 0.9$, t-test) (Fig. 4A,B). Moreover, the time course of the muscarine-induced depression of NMDA currents after rapamycin was not different from control, whereas after FK-506 the depression of NMDA currents tended to increase further with time (Fig. 3.4A).

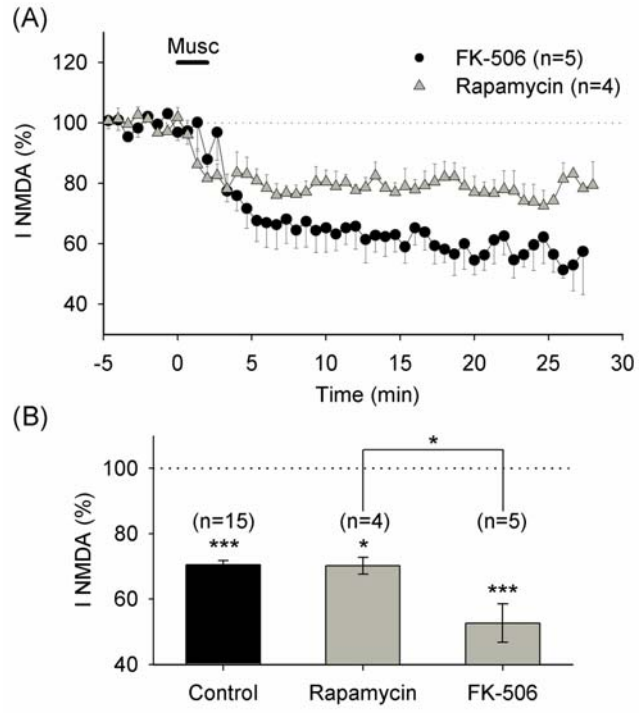


***Figure 3.4*. FK-506, a calcineurin antagonist, enhances the depression of NMDA currents by muscarine**. (A) Effect of FK-506 and rapamycin on the averaged time course of muscarine-induced depression. Note that muscarine-induced depression of NMDA currents after preincubation with rapamycin is indistinguishable from controls (see Fig. 1.) and significantly different from the effect of FK-506. (B) Pooled data showing that pretreatment with FK-506 accentuates the muscarine-induced depression.

The depression of NMDA current by muscarine in the presence of FK-506 was already significantly greater after 15 min compared to rapamycin (59.0 ± 5.4% with FK-506 versus 79.0 ± 6.0% with rapamycin, $p < 0.05$). These results suggest that in addition to the direct action on NMDA channels (Lieberman and Mody, 1994), calcineurin also modulates the activity of upstream components in the transduction pathway mediating the muscarinic depression of NMDA currents.

3.4.5 The Ca^{2+}/calmodulin Dependent Depression of NMDA Current is Mediated by a Protein Tyrosine Phosphatase(s)

Ca^{2+}/calmodulin has been shown to depress NMDA receptor function by binding to the C0 and C1 sites of the NR1 NMDA receptor subunit (Ehlers et al., 1996; Rycroft and Gibb, 2002a). In addition, calmodulin is likely to modulate NMDA responses indirectly by targeting signaling molecules associated with NMDA receptors. NMDA receptors are under the control of Src-family tyrosine kinases that upregulate and protein tyrosine phosphatases (PTPs) that downregulate their activity (Salter and Kalia, 2004). We therefore examined whether the muscarine-induced inhibition of NMDA currents is dependent on PTP activation by treating cells with pervanadate, a membrane permeable inhibitor of PTPs. Pervanadate (100 μM) induced a pronounced but reversible reduction of NMDA currents under baseline conditions (40.9 ± 9.5% of baseline, $n = 8$, $p < 0.001$), therefore we waited until a steady-state of the NMDA responses was achieved before applying muscarine. Furthermore, as pervanadate increases neuronal Ca^{2+} influx (P. Benquet, unpublished observations), nifedipine, an antagonist of L-type of Ca^{2+} channels, was added to the perfusate to exclude the possibility that Ca^{2+} will interfere with NMDA receptor modulation. In the continuous presence of pervanadate (100 μM) and nifedipine (10 μM), the effect of muscarine on NMDA currents was reversed from a depression to a potentiation (130.4 ± 6.8% of baseline, $n = 8$, $p < 0.005$) (Fig. 3.5A,C). Thus, the activation of tyrosine phosphatase is necessary for the depression of NMDA currents by muscarine.

Although the muscarinic depression of NMDA currents may be mediated by the tyrosine phosphatase-dependent dephosphorylation of the receptor, activation of tyrosine phosphatase can also reduce NMDA currents by directly inhibiting SFK activity (Nguyen et al., 2002). To discriminate between these two possibilities we

examined whether Src activation is required for the depression of NMDA currents by muscarine. Pretreatment of cultures with PP2 (10 μM, 15-20 min), a selective inhibitor of Src family tyrosine kinases, did not prevent the muscarine-induced depression of NMDA currents (67.5 ± 3.2% of baseline, $n = 5$, $p < 0.005$; Fig. 3.5B,C) indicating that the tyrosine phosphatase-dependent reduction of NMDA currents can occur independently of Src. These findings were further confirmed in experiments with SU6656, a further potent SFK inhibitor. Preincubation of slice cultures with 20 μM SU6656 blocked Src-dependent potentiation of NMDA currents in CA1 pyramidal cells ($n = 3$, data not shown) but failed to block the muscarine-induced depression of NMDA responses in CA3 (65.4 ± 1.1% of baseline, $n = 3$, $p < 0.001$; Fig. 3.5C).

We next examined whether protein kinase C (PKC), a major target of the M1 mAChR (Caulfield and Birdsall, 1998), is involved in the muscarine-induced depression of NMDA currents. We found that blocking PKC activity with the specific antagonist GF 109203X (2 μM, 20 min) does not alter the depression of NMDA currents (Fig. 3.5C) (69.5 ± 1.0% of baseline, $n = 4$, $p < 0.001$). Thus PKC does not appear to be involved in the pathway mediating the muscarinic depression of NMDA responses.

In a similar form of down-regulation of NMDA responses, stimulation of platelet-derived growth factor (PDGF) receptors induced a Ca^{2+}-dependent activation of cAMP-dependent protein kinase (PKA) that led to functional uncoupling of Src from the NMDA receptor (Lei et al., 1999). We therefore tested whether PKA is involved in the muscarine-induced depression of NMDA currents. After preincubation with the selective PKA antagonist H-89 (10 μM), muscarine still depressed NMDA responses (76.4 ± 1.8% of baseline, $n = 4$, $p < 0.05$), but less than in control (70.5 ± 1.3%; muscarine versus control: $p < 0.5$) (Fig. 3.5C).

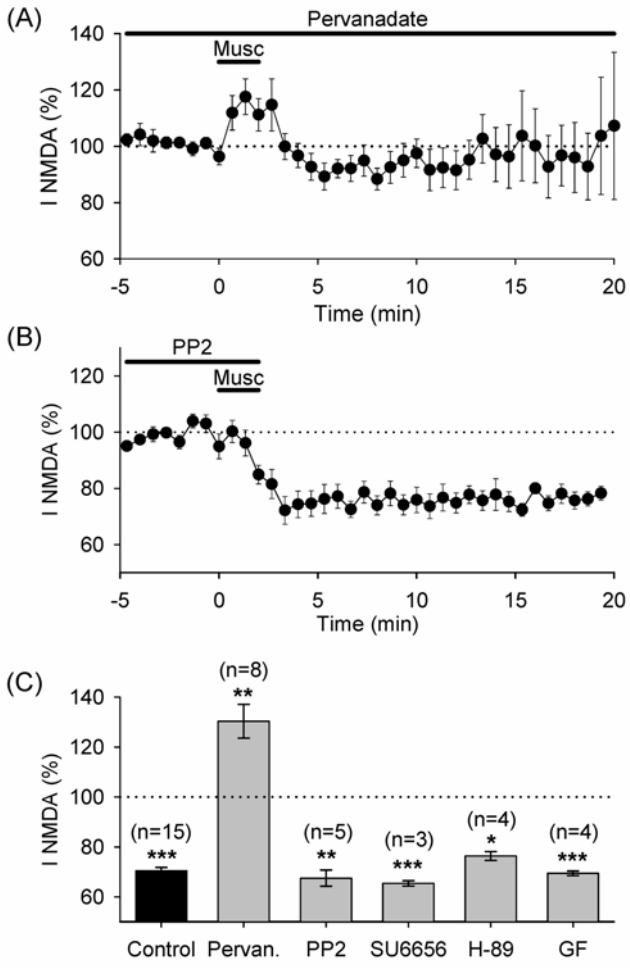


Figure 3.5. **An antagonist of protein tyrosine phosphatases converts the muscarine-induced depression of NMDA currents into a potentiation.** (A) Pretreatment with pervanadate converts the muscarine-induced depression into a potentiation. (B) Lack of effect of the Src inhibitor PP2 on the averaged time course of the muscarine-induced depression. (C) Pooled data showing effects of antagonists on NMDA-evoked currents.

3.5 Discussion

In this investigation we have demonstrated that stimulation of a neuronal muscarinic AChR can activate a protein tyrosine phosphatase thereby down-regulating NMDA responses. Numerous studies have focused on the potentiation of NMDA responses through activation of Src family protein tyrosine kinases, which phosphorylate specific residues on NMDA receptor subunits (Ali and Salter, 2001). Little is known, however, about the neuronal mechanisms that regulate the dephosphorylation of NMDA receptors. Stimulation of G protein-coupled receptors such as serotonergic receptors (Catarsi and Drapeau, 1997; Imbrici et al., 2000), muscarinic cholinergic receptors (Tsai et al., 1999), or metabotropic glutamate receptors (Canepari and Ogden, 2003; Ireland et al., 2004) increases tyrosine phosphatase activity in neurons. Furthermore, protein tyrosine phosphatases are closely associated with NMDA receptors and inhibition of tyrosine phosphatase activity increases metabotropic modulation of NMDA responses (Collett and Collingridge, 2004) and enhances unitary NMDA receptor currents in excised neuronal membranes (Wang et al., 1996). Activation of a tyrosine phosphatase via metabotropic glutamate receptors has also been shown to induce LTD, although the signal transduction pathway was not characterized in detail (Moult et al., 2002). Thus, it was not clear whether GPCRs can activate PTPs that target and modulate NMDA receptors.

Our finding that stimulation of mAChRs depresses NMDA currents in hippocampal CA3 pyramidal cells (Grishin et al., 2004) was unexpected in light of earlier reports describing an mAChR-induced potentiation of NMDA responses in CA1 pyramidal cells in acute slices (Markram and Segal, 1990a; Marino et al., 1998) and in dissociated cells (Lu et al., 1999), in striatal spiny neurons (Calabresi et al., 1998), and in auditory neocortical cells (Aramakis et al., 1999). This conflict was resolved by showing that mAChRs can couple to two divergent pathways that respectively potentiate or depress NMDA responses, and that the pathway that will be favored depends on ambient intraneuronal calcium levels (Grishin et al., 2004). Moreover, in certain cell types such as CA3 pyramidal neurons the coupling to the pathway resulting in the depression of NMDA responses is stronger. A number of analogous transduction mechanisms involving dual and opposing modulation of a common target by the same

receptor have been reported in the past. Thus, the activation of G_q-coupled muscarinic receptors induces biphasic modulation of $Ca_v2.3$ Ca^{2+} channels such that an initial inhibitory response mediated by $G_{\beta/\gamma}$ subunits is followed by a potentiation signaled through a PKC-dependent cascade (Bannister et al., 2004). A further example involves the dual modulation of NMDA receptors by the PDGF receptor, in which PDGF induces PKA-dependent depression of NMDA receptors, but can also activate a PKC and Src-dependent facilitating pathway (Lei et al., 1999). Of particular interest with respect to our findings is the coupling of the M1 AChR to both protein tyrosine kinases and protein tyrosine phosphatases targeting Kv1.2 potassium channels in the same cell (Tsai et al., 1999).

On the basis of the postsynaptic expression profile of mAChRs in hippocampal pyramidal cells (Levey et al., 1991) and the sensitivity of the muscarine-induced depression of NMDA currents to pirenzipine, we conclude that the M1 mAChR is mediating this response. Three of the five muscarinic receptor subtypes (M1, M3, M5) are coupled to G_q proteins, which target PLC leading to IP_3-induced intracellular Ca^{2+} release. Our experiments in which blocking G-protein function or depleting intracellular Ca^{2+} stores prevented the muscarinic depression of NMDA currents indicate that a pathway comprising an M1 receptor, G-protein activation and Ca^{2+} release represents the initial sequence of the transduction mechanism. Subsequently, Ca^{2+} calmodulin-dependent activation of a protein tyrosine phosphatase leads to a reduction of NMDA receptor function (see Fig. 3.6).

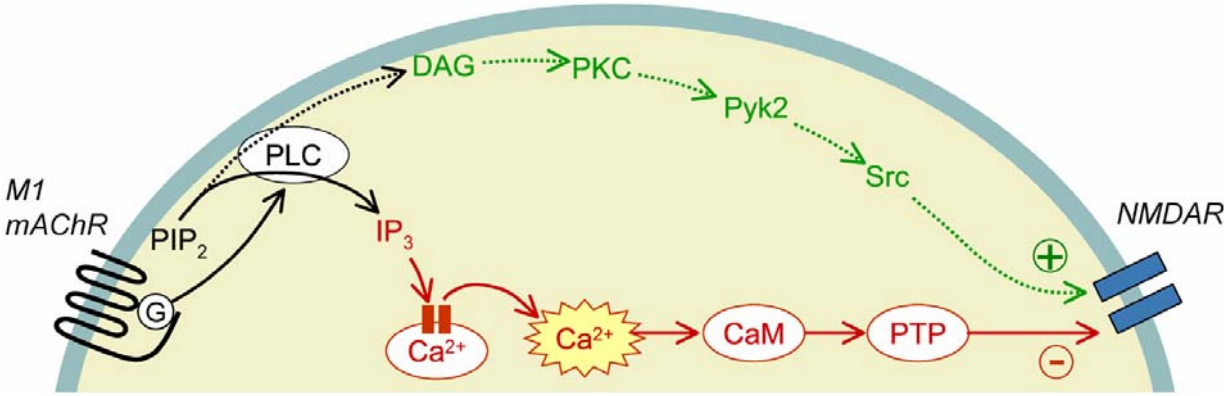


Figure 3.6. **A schematic diagram showing the Ca^{2+}-dependent pathway mediating the muscarine-induced depression of NMDA receptor function in CA3 hippocampal pyramidal cells.** Muscarine activates the M1 subtype of mAChRs, which induces G-protein-dependent release of calcium from intracellular stores. Subsequently, Ca^{2+} activates calmodulin, which in turn activates a tyrosine phosphatase(s) leading to downregulation of NMDA currents. This pathway opposes the Src-mediated enhancement of NMDA receptor function. Note that for reasons of clarity the scheme does not illustrate points of cross-talk between the two signaling cascades. The abbreviations used in the scheme: DAG, diacylglycerol; CaM, calmodulin; CaN, calcineurin; IP_3 , inositol triphosphate; PIP_2, Phosphatidylinositol-4,5-biphosphate.

3.5.1 Role of protein tyrosine phosphatases

Treating cells with pervanadate, a broad-spectrum inhibitor of tyrosine phosphatases, reversed the muscarine-induced depression of NMDA currents into a potentiation. At the same time, pervanadate strongly reduced NMDA receptor-mediated currents measured in the whole-cell mode. This result appears paradoxical, as pharmacologically inhibiting PTPs in excised patches increases NMDAR channel gating (Wang et al., 1996). However, blocking PTPs has been shown to decrease NMDA responses in intact cells (Lei et al., 2002) reflecting the fact that at least five protein tyrosine phosphatases are known to upregulate the activity of Src (for review see (Paul and Lombroso, 2003; Salter and Kalia, 2004)). Thus, this discrepancy can be explained by the observation that in excised membrane patches only PTPs directly associated with the NMDA receptor are blocked, whereas in whole-cell recordings PTPs that upregulate Src will be blocked as well. We therefore suggest that the decrease of whole-cell NMDA currents by pervanadate may be due to the inhibition of PTPs that tonically upregulate Src family kinases. However, it is possible that blockade of PTPs directly upregulating NMDARs, e.g. the phosphatase PTEN, (Ning et al., 2004), may in part mediate the effect of pervanadate on baseline NMDA currents. Thus, in our experiments we appear to observe both effects of pervanadate, a reduction of basal NMDA currents by blocking PTP-dependent Src activation, and an uncoupling of the muscarinic inhibitory pathway targeting the NMDA receptors. Interestingly, inhibition of Ca^{2+} release or calmodulin completely blocked the muscarine-induced depression, but in contrast to PTP blockade, did not reverse the response to a significant potentiation. These results can be explained by considering that pathways leading to Src-dependent potentiation of the NMDA response require Ca^{2+} for the activation of PKC (Parker and Murray-Rust, 2004) and the Ca^{2+}/calmodulin-dependent activation of Pyk2 that targets Src (Heidinger et al., 2002). The conversion of the muscarine-induced depression into a potentiation of NMDA responses after pretreatment with pervanadate indicates that even under conditions that favor depression, tyrosine kinases can still be transiently activated (Fig 5A). However, we observed no additive effect on the depression of NMDA currents when SFKs were blocked in experiments with PP2 and SU6656. This finding may indicate that kinase activation is transient (Fig. 5A) and

precedes the activation of tyrosine phosphatases (Fig. 1B), or that the effect of tyrosine phosphatases on NMDA receptors is already saturated under our recording conditions.

We found that pharmacological blockade of calcineurin enhanced the depression of NMDA responses by muscarine, which seems paradoxical as calcineurin reduces the duration of NMDA channel open time (Lieberman and Mody, 1994). However, apart from its action on NMDA receptor channels, calcineurin also reduces the depressing effect of calmodulin on NMDA single channel activity (Rycroft and Gibb, 2004). Thus, the enhancement of the muscarinic inhibition of NMDA responses may reflect a modulatory effect of calcineurin on upstream components in the signaling pathway targeting NMDA receptors.

Distinct PTPs can either increase or decrease the activity of SFKs. For example, PTPα leads to Src activation, whereas striatal-enriched tyrosine phosphatase (STEP) dephosphorylates and inhibits the SFK Fyn (Nguyen et al., 2002). Thus, PTPs are able to reduce NMDA receptor responses by inhibiting SFKs or by dephosphorylating the receptor (Paul and Lombroso, 2003). To distinguish between these two possibilities we checked if Src activity is necessary for the muscarine-induced depression of NMDA currents. However, treatment with PP2 or SU6656, selective SFK inhibitors, did not significantly modify the muscarinic depression of NMDA current. Another mechanism that can interfere with Src-dependent phosphorylation of NMDA receptors is the uncoupling of Src from NMDA receptors through activation of PKA by treating hippocampal cells with PDGF (Lei et al., 1999). We found that H-89, a specific PKA antagonist, caused a small but significant reduction in the muscarinic inhibition of NMDA current. In contrast, inhibition of PKA completely blocked the depression of NMDA currents by PDGF (Lei et al., 1999). The small reduction in the muscarinic depression of NMDA current after PKA inhibition may reflect a disruption of the tonic interaction between PKA and the NMDA receptor via the scaffolding protein Yotiao (Westphal et al., 1999). Taken together these results suggest that muscarinic depression of NMDA responses is mediated by PTPs acting not via inhibition of SFKs, but possibly through a direct dephosphorylation of the NMDA receptor.

3.5.2 Multi-Pathway Signaling Networks

Previous studies have established that G_q-coupled GPCRs trigger a transduction process that activates Src family tyrosine kinases leading to the phosphorylation and upregulation of NMDA receptor function in CA1 and CA3 hippocampal pyramidal cells (Lu et al., 1999; Benquet et al., 2002; Heidinger et al., 2002). Our study provides evidence for a parallel but antagonistic pathway also initiated by the stimulation of a G_q-coupled GPCR that activates a PTP leading to the downregulation of NMDA receptor function in CA3 hippocampal pyramidal cells. We therefore propose that two mutually opposing signaling mechanisms – the positive one mediated by a tyrosine kinase, the negative one by a PTP – can be triggered by activation of the same G_q-coupled GPCR to modulate the gain of NMDA receptors. Our earlier data indicate that intracellular Ca^{2+} concentration determines whether M1 muscarinic receptors couple to the negative or positive pathway, allowing the cell to adapt to physiological requirements (Grishin et al., 2004). Thus we have shown that G_q-coupled receptors, in addition to their established role in Src activation, also control NMDA receptor function by intracellular feed-forward inhibition that restricts or even overcomes Src signaling. We suggest that dual antagonistic transduction pathways activated by the same receptors may be a widespread phenomenon representing a further mechanism for cells to control precisely the phosphorylation state of target proteins.

Chapter 4

Conclusion

4. Conclusion

4.1 Future research prospects & outlook

Previous investigations on NMDA receptor modulation have revealed that Src family kinases are the point of convergence for multiple signaling pathways, considered as the "hub" of NMDAR regulation (Salter and Kalia, 2004). However, although numerous pathways have been shown to upregulate SFKs, little is known about how NMDAR function can be specifically inhibited. The most explored mechanism of inhibition of NMDARs is desensitization, which is a non-specific process. Some forms of desensitization are calcium- and calmodulin-dependent (Kotecha and MacDonald, 2003). In contrast to previous work we have found that G_q-coupled metabotropic receptors are linked to both Src and a Src-independent inhibitory pathway that activates tyrosine phosphatase and thus counterbalances Src action. The next challenge will be the molecular identitication of the tyrosine phosphotase(s) mediating the muscarine-induced depression of NMDA currents. Because of a lack of specific PTP antagonists and the probable participation of multiple tyrosine phosphatases in the modulation of NMDAR and SFK activity, it is likely that the solution to this problem will require the implementation of molecular biological and biochemical approaches.

Thus NMDAR receptor function is critical for normal CNS operation and its dysfunction leads to pathological states, therefore elucidation of mechanisms modulating NMDARs may be a key to understanding glutamatergic transmission and plasticity and can greatly advance the field of rational drug design.

4.2 How many pathways are there?

In CA3 hippocampal neurons, mGluRs have been shown to modulate NMDAR function both by a PKC/Pyk2/Src pathway as well as a G-protein-independent mechanism (Benquet et al., 2002). Our preliminary data indicate the presence of a similar pathway mediating a G-protein-independent potentiation of NMDAR function in CA3 pyramidal neurons following the stimulation of muscarinic receptors. Specifically, G-protein blockade combined with Ca^{2+} buffering (10 mM EGTA in the

patch pipette) results in a significant potentiation of NMDA currents in CA3 neurons (122.9 ± 5.9% of baseline, $p < 0.05$, $n = 5$) (Fig. 4.1). This result suggests that mAChRs-induced potentiation of NMDA currents is in part mediated by a G-protein-independent mechanism.

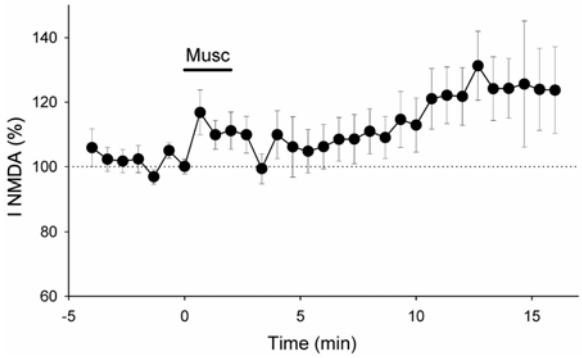


Figure 4.1 **G-protein blockade and Ca^{2+} buffering (10 mM EGTA) results in a muscarine-induced potentiation of NMDA currents.** The averaged time course of NMDA responses is presented.

This finding is further supported in experiments following blockade of PKC, an enzyme mediating G-protein-dependent activation of Src. We found that after preincubation of slice cultures with a PKC inhibitor GF 109203x (2 μM, 20 min preincubation) muscarine induced a biphasic modulation of NMDA currents – a potentiation, followed by a depression of NMDA currents (Fig 4.2).

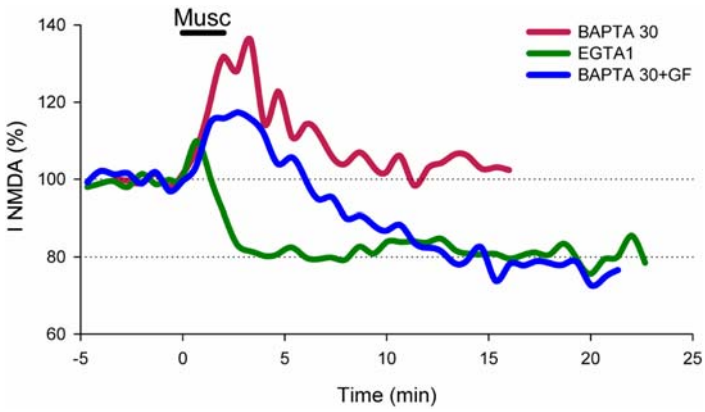


Figure 4.2 **Multiple modulatory pathways interact to fine-tune the gain of NMDA receptors.** Time course of muscarine-induced NMDAR modulation recorded under various conditions allowing full potentiation, full depression, and a biphasic response due to pharmacological blockade of one of the modulatory pathways. Note that blocking PKC under conditions favoring potentiation results in a G-protein independent enhancement of NMDA currents followed by G-protein dependent depression.

The potentiation was attenuated compared to control conditions (125.5 ± 6.4%, $p < 0.5$, $n = 5$) but was not significantly different from the potentiation induced after the G-protein blockade with EGTA 10 mM ($p > 0.78$, t-test). The subsequent depression

(69.6 ± 3.6%, $p < 0.005$, $n = 5$) was not significantly different from control ($p > 0.8$, t-test). The biphasic appearance of the response demonstrates a sequential activation of the G-protein-independent upregulating cascade, followed by the G-protein-dependent Ca^{2+}-sensitive depression of NMDA responses (Fig. 4.2). The family of related curves in Fig. 4.2 depicts the interplay of different modulatory pathways and confirms the presence of the G-protein-independent pathway modulating NMDARs and activated by mAChRs.

4.3. Changing Concepts of Metabotropic Signaling

Paradigms of metabotropic receptor signaling are changing to accommodate new experimental data revealing an unexpected complexity and diversity of intracellular signaling cascades. It has been established that distinct neuromodulatory systems, which were previously thought to be independent, transmit an array of divergent intracellular signals some of which exhibit convergence onto common targets. For instance, both growth factors and classical neurotransmitters have been shown to activate MAP kinase, which results in altered gene expression and adaptation of the cell to environmental changes (for review see (Luttrell and Luttrell, 2003). In this example a neurotransmitter acts as a growth factor. Furthermore, BDNF, a growth factor, has been shown to modulate neuronal excitability by direct gating of $Na_v1.9$ ion channels, thereby acting as a classical neurotransmitter (reviewed by (Rose et al., 2004). Thus receptors can produce a broad spectrum of signals with a considerable overlap.

My work shows that the same holds true within a single signaling system. We were able to demonstrate that neurotransmitter-activated G-protein-coupled receptors signal via multiple divergent pathways with points of cross-talk and common downstream targets. Thus, mAChR-induced modulation of NMDAR function appears to involve at least three divergent pathways converging on the NMDA receptor. Consequently, our results suggest that metabotropic receptor signaling is more complex than previously considered and relies on the activation of a “signaling network” rather than a “signaling pathway”.

Although a simultaneous activation of antagonistic pathways modulating ion channels by controlling tyrosine phosphorylation has been shown before (Tsai et al., 1999), we report an interesting mechanism regulating the activation of these pathways

relative to each other. Our results demonstrate that calcium plays an essential role in the muscarine-induced regulation of NMDA currents.

One of our most important findings was that the negative and positive pathways modulating NMDAR activity exist both in CA1 and CA3 pyramidal neurons but nonetheless mAChRs mediate mainly a potentiation in CA1 and a depression in CA3 pyramidal cells. Thus, muscarinic receptors in CA1 and CA3 are coupled to their respective downstream signaling cascades in a cell type-specific manner. CA1 and CA3 pyramidal cells are known to subserve different functions in the hippocampus and are therefore specialized accordingly differing in morphology and protein expression patterns. We propose that this cell-specific coupling of metabotropic receptors may reflect distinct functions of these two cell types in the hippocampal circuitry.

Chapter 5

5. Attached manuscript

Neuronal expression of transgenic adenosine kinase in mouse brain leads to enhanced spontaneous locomotor activity

Abbreviated title: Transgenic adenosine kinase expression

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Abstract

Adenosine kinase (ADK) is the key enzyme for the regulation of tissue adenosine levels. In brain, ADK expression is normally confined to a subpopulation of astrocytes and an up-regulation of ADK in these cells has been associated with astrogliosis and chronic seizure activity. To investigate the consequences of cell-type specific ADK expression on brain activity and epileptogenesis, a mouse model was developed in which endogenous astrocytic ADK levels were enhanced by ubiquitous transgene-driven expression of ADK. Working with an *Adk* knockout background an *Adk* transgene under the control of a ubiquitin promoter was introduced. As a result, ADK-expression was reconstituted in all organs analyzed and led to a phenotypic rescue of the otherwise lethal ADK-deficiency. The observed lack of endogenous ADK in astrocytes and the ubiquitous overexpression of ADK throughout the whole brain, with particularly high levels in pyramidal neurons of the hippocampus was associated with a loss of hippocampal paired pulse facilitation indicating a deficit in adenosine-mediated presynaptic inhibition. Behaviorally, the mutants displayed increased motor activity but, despite an apparent lack of adenosinergic tone, they displayed no spontaneous seizures. However, mutants with intrahippocampal injections of kainic acid displayed astrogliosis and seizure activity, but failed to exhibit overexpression of ADK when tested four weeks after drug injection. Thus, we conclude that overexpression of ADK in epileptic hippocampus is primarily a consequence of seizure activity or astrogliosis, and ultimately may lead to an aggravation of seizures in chronic epilepsy.

Introduction

The purine ribonucleoside adenosine is an important modulator with mostly inhibitory effects on neuronal activity (Dunwiddie and Masino, 2001). Thus, activation of adenosine A_1 and A_{2A} receptors (Fredholm et al., 2001) by selective agonists (Huber et al., 2002; Gouder et al., 2003) or by adenosine delivered by implanted cells (Huber et al., 2001; Boison et al., 2002a) promotes seizure suppression. Conversely, adenosine receptor antagonists such as caffeine stimulate the CNS (Fredholm et al., 1999). On the basis of its low K_M for adenosine, adenosine kinase (ADK, EC 2.7.1.20) is considered to be the key enzyme for the regulation of adenosine levels (Mathews et al., 1998) and inhibitors of ADK, therefore, produce a substantial elevation in adenosine levels *in vitro* (Pak et al., 1994). Accordingly, pharmacological inhibition of ADK increases adenosine A_1-receptor mediated presynaptic inhibition in hippocampal slices (Pak et al., 1994) and suppresses seizures in various models of epilepsy (Wiesner et al., 1999; Kowaluk and Jarvis, 2000). In contrast, inhibition of adenosine deaminase in brain has little or no influence on the concentration of extracellular adenosine (Pak et al., 1994; Huber et al., 2001). As a consequence, ADK is crucial in setting adenosine levels and thereby regulating brain activity. Furthermore, a complete lack of ADK in knockout mice is associated with early postnatal microvesicular hepatic steatosis and death (Boison et al., 2002b).

Normally, brain activity is under a tonic inhibitory adenosinergic tone resulting from low basal expression of ADK in a subpopulation of astrocytes, which is distributed evenly throughout the whole brain (Gouder et al., 2004). Recently, an overexpression of ADK in astrocytes of epileptic hippocampus was shown to parallel astrogliosis and chronic seizure activity and thus may contribute to epileptogenesis by lowering the endogenous inhibitory adenosinergic tone in this critical brain area (Gouder et al., 2004). In this previous study, the question arose whether astrogliosis, a hallmark of temporal lobe epilepsy, and overexpression of ADK is a consequence or a cause of seizure activity. To address at a molecular level the potential relationship between ADK overexpression in astrocytes of epileptic hippocampus and epilepsy-associated astrogliosis, we created a mouse model in which the endogenous expression of ADK in astrocytes was abolished and replaced by global transgene-driven expression of ADK. This was achieved by introducing an *Adk* transgene under the control of a human ubiquitin promoter into an *Adk* knockout (*Adktm1*$^{-/-}$) background. The shift in ADK

expression resulted in a loss of hippocampal paired-pulse facilitation, an increase in motor activity, but not in spontaneous seizure activity. However, mutants with intrahippocampal injections of kainic acid displayed astrogliosis and seizure activity, comparable to wild-type control animals, but no overexpression of ADK four weeks after drug injection. Thus, we conclude that overexpression of ADK in epileptic hippocampus is primarily a consequence of seizure activity and astrogliosis, and ultimately may lead to an aggravation of seizures in chronic epilepsy.

Materials and Methods

Generation of adenosine kinase transgenic mice. *Adktm1*$^{-/-}$-mice were created by homologous recombination of the *Adk* gene in embryonic stem cells as described (Boison et al., 2002b). For the generation of *Adk* transgenic animals an 1865 bp full length *Adk* cDNA was generated from mouse liver using a Marathon cDNA Amplification Kit (Clontech, Palo Alto, Ca.) and the internal *Adk* PCR primers 5'-CCAATGCATCCGAAGAACGTTGCTGC-3' (5' RACE) and 5'-GTGAACTCGAAGAGGCAGAGGACCG-3' (3'-RACE). The cDNA was inserted into an EcoRV site of the pBluescriptIIKS+^{TM} vector (Stratagene, La Jolla, Ca.). The insert was sequenced and identified as a full length mouse *Adk* cDNA being homologous to the short isoform (38.7 kDa) of *Adk* described in humans (McNally et al., 1997). After subcloning into the bacterial expression vector pQe60 (Qiagen, Hilden, Germany) the mouse *Adk* cDNA was expressed in E. coli SM15 cells (Qiagen) and found to display ADK activity (Boison et al., 1999). For the generation of an ubiquitously expressed *Adk*-specific transgene the *Adk* cDNA was cloned into the multiple cloning site of a transgene expression vector containing a human ubiquitin promoter and the splice and poly(A) sequences of SV40 (Schorpp et al., 1996). In a last cloning step the *Adk* expression cassette was flanked with loxP sites. The linearized vector was purified and injected into the pronucleus of fertilized mouse eggs derived from *Adktm1*$^{+/-}$ breedings. Three independent lines of *tgUbiAdk:Adktm1*$^{+/-}$ mice were established in a mixed 129/JEms and C57BL/6 background. Two of these lines were bred to homozygousity of the knockout allele, giving rise to two different lines (line 888 and line 890) termed *tgUbiAdk:Adktm1*$^{-/-}$ (*Adk* transgene expression in *Adk* knockout background). It is important to note that in both lines (888 and 890) the introduction of the *UbiAdk* transgene into the *Adk* knockout background provided a complete rescue of this lethal mutation. *TgUbiAdk:Adktm1*$^{-/-}$ mice are viable, reproduce and have no overt abnormality. All mice used in this study were maintained on a mixed 129/JEms and C57BL/6 background.

Genotyping of mutant mice. For the analysis of *Adk*-mutant mice, PCR reactions were performed with the following sets of primers: The endogenous *Adk*-locus was analyzed in individual reactions containing a mix of the 3 primers o107, 5'-CTC ACT TAA GCT GTA TGG AGG TGA CCG-3' (sense primer specific for wild type *Adk*); o108, 5'-AGT CAC AGA TGC ATC TGC AGA GGT GAG-3' (antisense primer

specific for wild type *Adk*); and o109, and 5'-ACT GGG TGC TCA GGT AGT GGT TGT CG-3' (antisense primer specific for null allele). PCR-primers used for detection of the *Adk* transgene were o141, 5'-GGA AGC TGC CAC TTT TGC TAG AGA GC-3' and o142, 5'-GGA ACG CTC AGG ATG TGG TCA CTG C-3'. Amplifications were carried out as described previously (Fedele et al., 2004). The combination of primers o107 with o108 gave rise to 640 bp products indicative of a wild type *Adk* allele, whereas the combination of primers o107 with o109 gave rise to 840 bp products indicative for the *Adk*-knockout allele. The combination of primers o141 and o142 gave rise to 420 bp products being indicative for the transgene.

The zygousity of the transgene was determined in a TaqMan™ real time PCR analysis as follows: Individual TaqMan™ PCR reactions were performed in duplicates with the ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) in a reaction volume of 25 µl, containing 1x universal master mix (Applied Biosystems, Rotkreuz, Switzerland), 300 nM of each forward and reverse primer and 100 nM fluorescent probe (Microsynth, Balgach, Switzerland). The temperature cycling program was set at 2 min initial incubation at 50°C, followed by 10 min at 95°C and 40 PCR cycles with 15 s at 95°C and 1 min at 60°C. For the quantification of the transgene, the following transgene specific primers were used: tg-fwd (5'-ACT TGT GCT GCG TGC ATC A-3'), tg-rev (5'-TAA CAA TTG GCG GCA GCA A-3'), and a 5'-FAM and 3'-TAMRA labeled fluorogenic TaqMan™ probe (5'-FAM- CAA CGA GGG ACC TGT TGC CAC CA -3'-TAMRA). After completion of the PCR reactions a threshold of the fluorescence intensity was set within the linear phase of the amplifications. The cycle number, in which this threshold was crossed by the active reporter fluorescence was determined (cycle threshold, CT). The zygousity was determined by comparison of the CT values of the respective *tgUbiAdk*-allele with the CT values from the amplification of the *Adk* knockout allele, which was used as an internal reference gene (two allele copies in *tgUbiAdk:Adktm1*$^{-/-}$ mice). The knockout specific primers and probes were specific for the EGFP containing gene insertion cassette: Adk-ko-fwd (5'-GCC GTC TTT TGG CAA TGT G-3', Adk-ko-rev (5'-CCC CTA GGA ATG CTC GTC AA-3'), and a 5'-FAM and 3'-TAMRA labeled fluorogenic TaqMan™ probe (5'-FAM- CCG GGA AAC CTG GCC CTG TCT T -3'-TAMRA). With this strategy, mice, homozygous for the transgene (2 copies) were selected to

breed *tgUbiAdk:Adktm1*$^{-/-}$ mice to homozygousity. Unless otherwise mentioned, all *tgUbiAdk:Adktm1*$^{-/-}$ mice used in this study were homozygous for the transgene.

Western Blot analysis. Aqueous extracts from whole brains, livers, lungs, kidneys, and hearts derived from adult *tgUbiAdk:Adktm1*$^{-/-}$ and wild type mice were prepared by homogenizing and solubilizing the tissue in two volumes of a 5 mM EDTA solution and by removing unsolubilized material by centrifugation (100,000 x g, 15 min., 4°C). The protein content in the supernatants was determined using a commercial Bradford assay (Bio-Rad, Hercules, Ca.). 15 µg of each supernatant was separated on a SDS/10%PAGE gel and blotted onto a nitrocellulose membrane according to standard procedures. The blots were probed for 1 hour with a 1:6000 dilution of a polyclonal rabbit antiserum against ADK (Boison et al., 2002b; Gouder et al., 2004) in 5% blocking reagent in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20) in H_2O). After washing (5 x 5 min in TBST), blots were then probed with a peroxidase-linked anti-rabbit IgG and bands were visualized with a commercial enhanced bioluminescence detection method (ECL- kit; PerkinElmer Life Sciences, Boston, Ma.).

Behavioral analysis. Spontaneous locomotor activity was assessed in individual circular cages equipped with four automated equidistant photocells (Imetronic, Pessac, France). Male mice, which were housed in individual cages, were adapted to the testing room under reversed 12-h light-dark cycle (light onset: 8 pm) conditions for 10 days before the experiment.

Locomotor activity was recorded continuously over a period of four days. Data were collected during the last 3 days for statistical analysis. Three days after completion of the experiment the mice were sacrificed by decapitation to obtain samples for ADK activity measurements. All animal experimentation was approved by the local animal welfare authorities.

Adenosine kinase enzyme activity assay. Whole brains were carefully dissected from freshly killed adult *tgUbiAdk:Adktm1*$^{-/-}$, *Adktm1*$^{+/-}$, and wild type mice (the same mice which were used for the behavioral study). Brains were processed individually at 4°C by one 5-second burst of ultrasound homogenization in 1 ml of 50 mM NaCl, 1 mM Na_2HPO_4, 5 mM EDTA, and 10 mM Tris, pH 7.6. The homogenates were then centrifuged at 100000g for 10 min. To remove nucleotides and nucleosides from the supernatants, 100 µl of each sample were applied to a Micro Bio-Spin column, which

was filled with Dowex 1x8 (Fluka, Buchs, Switzerland) equilibrated in lysis buffer. After centrifugation at 5000 g for 2 min, the flow-through was diluted with 300 µl FL-AAB. 5 µl of each diluted sample were used for adenosine kinase activity determinations with an enzyme-linked bioluminescent assay according to the following principle: An excess of adenosine, added to the sample, is phosphorylated by adenosine kinase to AMP, which is then phosphorylated further to ATP by an excess of recombinant GTP:AMP phosphotransferase (own production) and pyruvate kinase (Roche, Rotkreuz, Switzerland). The resulting ATP increase is then quantified with a luciferase assay (ATP Bioluminescent Assay Kit, Sigma). 5 µl of each diluted sample containing an unknown activity of ADK was added to 20 µl of a solution containing 5 mU/ml GTP:AMP phosphotransferase, 2 U/ml pyruvate kinase, 400 µM GTP, 400 µM phosphoenolpyruvate, 10 µM *erythro*-9-(3-(2-hydroxynonyl))adenine, and 100 µM adenosine in 30% Adenosine 5'-phosphate Assay Mix (v/v) in FL-AAB in 96-well microtiter plates. The increase of ATP from the ongoing reaction was detected and recorded for 15 min at room temperature in a luminometer (Microlumat plus LB96V, EG&G Berthold). ADK activity was determined as an increase of relative light units per time. ADK activity values were normalized to endogenous lactate dehydrogenase (LDH) activity, which was taken as an internal standard. LDH activity was determined with a commercial assay kit according to the manufacturer's protocol (Roche, Rotkreuz, Switzerland). The assay was validated by plotting a standard curve of different ratios of samples from wild-type mice mixed with those from adenosine kinase knockout mice. Statistical analysis of paired groups was performed by Mann-Whitney tests.

Histological analysis of mouse brain. Adult *tgUbiAdk:Adktm1*$^{-/-}$ and wild type mice were transcardially perfused with 4% paraformaldehyde and 15% saturated picric acid solution in phosphate buffer (0.15 M, pH 7.4). The brains were then post fixed in the same fixative at 4°C for 6 hours and cryoprotected in 10% DMSO in PBS (v/v) before being cut into 40 µm sagittal or coronal sections using a sliding microtome. For the immunohistochemical detection of ADK, brain sections were incubated overnight at 4°C with primary anti-ADK antiserum diluted 1:5,000 in Tris-Triton pH 7.4 containing 2% normal goat serum and 0.2% Triton X-100. The sections were then washed 3x10 minutes in Tris buffered saline (TBS) at pH 7.4, incubated for 30 minutes with biotinylated goat anti-rabbit antibody diluted 1:300 in Tris-Triton pH 7.4 containing 2% normal goat serum, washed again 3 times in TBS then incubated for 20-30 minutes with

an Avidin:Biotin enzyme complex (Vectastain Elite Kit; Vector Laboratories, Burlingham, Ca., USA). After washing again three times with TBS the tissue antigen was localized by incubation with diaminobenzidine hydrochloride (Sigma, St. Louis, Mo., USA), which acts as a chromogen. The sections were then mounted on gelatin-coated slides, air-dried, and coverslipped. The immunoperoxidase stained sections were analyzed with a Zeiss AxioCam microscope (Zeiss, Jena, Germany) equipped with bright field microscopy, using a high-resolution digital camera.

Immunofluorescence staining. In order to assess the kainic acid induced degree of astrogliosis, an immunofluorescence staining was performed on coronal brain sections from naïve control wild-type or *tgUbiAdk:Adktm1*$^{-/-}$ mice and from wild-type or *tgUbiAdk:Adktm1*$^{-/-}$ mice sacrificed 4 weeks after the injection of KA. Astrogliosis was evaluated by immunofluorescence of a monoclonal mouse antibody against the astrocytic marker glial fibrillary acidic protein (GFAP) (MAB360, Chemicon International, Temecula, Ca., USA).

Brain sections were washed under agitation in phosphate buffered saline (PBS) for thirty minutes, changing the solution three times, and were then incubated overnight at 4°C in a solution containing the primary GFAP antibody diluted 1:5000 in Tris-Triton pH 7.4 which contained 2% normal goat serum and 0.2% Triton X-100. After three 10 minute washes in Tris-Triton pH 7.4, sections were incubated for 30 minutes at room temperature in a solution containing the secondary antibody goat anti-mouse conjugated to Cy2 (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa., USA) to detect GFAP. The secondary antibody was diluted 1:200 in Tris-Triton containing 2% NGS. Sections were washed a further three times for 10 minutes, mounted on gelatinized slides and air-dried before being coverslipped with Dako fluorescent mounting medium (Dako Corporation, Carpinteria, Ca., USA). To verify the specificity of the monoclonal antibody, control stainings were performed with secondary antibody only. The control stainings were devoid of localized immunofluorescence (data not shown).

High-resolution images were analyzed by laser scanning confocal microscopy (LSM510-Meta, Zeiss, Jena, Germany). Stacks of 12-20 images spaced by 0.4-0.6 μm were color-coded. The digitized images were processed with Imaris software (Bitplane, Zurich, Switzerland).

Hippocampal slice cultures and in vitro electrophysiology. Hippocampal slice cultures were prepared from 6-day-old mice and maintained using the roller-tube technique, as described previously (Gahwiler et al., 1998). After 3-4 weeks *in vitro*, cultures were transferred to a 1 ml recording chamber that was perfused continuously with extracellular solution (1.5 ml/min, 30°C) containing (in mM): 137 NaCl, 2.7 KCl, 11.6 $NaHCO_3$, 0.4 NaH_2PO_4, 2 $MgCl_2$, 3 $CaCl_2$, 5.6 D-glucose, 0.001% phenol red, pH 7.4, ~305 mOsm. Whole-cell voltage clamp recordings were obtained from visually identified CA3 pyramidal cells at -70 mV using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Signals were filtered at 2 kHz and digitally recorded using pClamp 7 software (Axon Instruments). Patch pipettes (3-5 MΩ; series resistance 4-15 MΩ) were filled with: (in mM) 130 $CsCH_3O_3S$, 9.91 CsCl, 10 HEPES, 10 EGTA, 1 picrotoxin, 4 Mg-ATP, 0.4 Na-GTP, pH 7.2, ~288 mOsm. Liquid junction potentials (-12.5 mV) were corrected for. To stimulate mossy fibers a monopolar glass electrode was placed in the hilus directly adjacent to the granule cell layer. Paired pulses with an interstimulus interval of 40 ms (300 μs, 0.5 – 50 μA, 0.05 Hz) were used for stimulation. The paired-pulse ratio was calculated as the amplitude of the second EPSC divided by the amplitude of the first. To reduce polysynaptic activity the Ca^{2+} and Mg^{2+} concentrations in the extracellular solution were increased to 3 and 2 mM, respectively. To obtain pure excitatory EPSCs, GABAergic contamination was minimized by including 1 mM picrotoxin into the patch pipette and adjusting the Cl^- reversal potential to -70 mV by modifying the $[Cl^-]_i$ in the intracellular solution according to the Nernst equation.

Kainate induced epileptogenesis. Experiments were conducted on male *tgUbiAdk/Adk*$^{-/-}$ (n = 7) and wild-type control mice of the same genetic background (n = 4), which were housed in individual cages in a 12-h light–dark cycle (light on from 07:00 to 19:00) with food and water provided *ad libitum*. All animal procedures were conducted in accordance with the regulations of the local animal welfare authority. All efforts were made to minimize animal suffering and to reduce the number of animals. Under general anesthesia using equithesin (4 ml/kg i.p.) the experimental mice were stereotactically injected with 50 nl of a 20 mM solution of kainic acid (KA) in 0.9% NaCl (i.e., 1 nmol KA) into the right dorsal hippocampus (coordinates with bregma as reference: anteroposterior (AP) = -1.5, mediolateral (ML) = -1.8, dorsoventral (DV) = -1.9 mm) using a stainless steel cannula (outer diameter, 0.28 mm) connected to a 0.5 μl

microsyringe (Hamilton, Bonaduz, Switzerland). Each injection was performed over a period of 1 min. At the end of the injection, the cannula was left in place for an additional period of one minute to limit reflux along the cannula track. Unless noted otherwise, the mice were transcardially perfused 4 weeks after KA injection and processed for ADK immunohistochemistry. In addition to ADK, GFAP was used to monitor astrogliosis and NeuN to verify expression of transgenic ADK in neurons.

Electroencephalographic recordings. **To monitor seizure activity** all KA-injected animals (n = 7 *tgUbiAdk/Adk*$^{-/-}$; n = 4 wt) and, in addition, non-treated *tgUbiAdk/Adk*$^{-/-}$ (n = 17) and wild-type (n = 9) mice as controls were implanted with a bipolar electrode inserted into one hippocampus and a monopolar reference electrode, which was placed over the cerebellum. The bipolar electrode, formed of two twisted enamel insulated stainless steel wires (diameter, 170 µm, distance between the tips, 0.4 mm), was inserted into the right dorsal hippocampus (coordinates with bregma as reference: AP = -1.5, ML = -1.8, DV = -1.9 mm). The monopolar reference electrode, made of the same enamel insulated stainless steel wire (diameter, 250 µm; Wire pro, Farnell, France), was inserted in the skull so that only the tip (0.5 mm) protruded onto brain tissue. In addition to the hippocampal and reference electrodes 6 *tgUbiAdk/Adk*$^{-/-}$ mice and 6 wild-type control mice were also equipped with 2 monopolar electrodes to record cortical EEG activity. The electrodes were fixed to the skull with cyanoacrylate and dental acrylic cement. When the mice recovered from anesthesia, seizure activity was recorded using an EEG recording chamber placed in a Faraday cage. EEG activities of freely moving animals were recorded using a digital acquisition computer-based system (MP100WSW System, Biopac Systems Inc., Santa Barbara, CA, USA; 10 channels, sampling rate 200 Hz). Before starting the EEG recordings a period of one hour was allowed for the habituation of the animals to the test cage. The recordings were taken for a period of 9 h during the resting phase of the animals (9:00 – 18:00).

Results

Insertion of an *Adk*-transgene provides genetic rescue for ADK-deficiency

To study cell type specific functions of brain adenosine kinase (ADK), we shifted the expression of ADK from selected astrocytes to a widespread expression pattern encompassing both neurons and astrocytes by introducing an ubiquitously expressed *Adk* transgene into an ADK-deficient host mouse line (*Adktm1*$^{-/-}$) (Boison et al., 2002b). This procedure also resulted in genetic rescue of the lethal *Adktm1*$^{-/-}$ phenotype. We constructed a transgene (*tgUbiAdk)* of an *Adk* cDNA (Boison et al., 1999) corresponding to the short (38.7 kDa) brain specific isoform of the enzyme (McNally et al., 1997; Gouder et al., 2004) under the control of the human ubiquitin promoter (Schorpp et al., 1996) and containing the splice and poly(A) sequences of SV40 (Fig. 1*A*). The construct was flanked by loxP sites to allow future tissue specific excision of the transgene.

The transgene was injected into pronuclei of fertilized mouse eggs derived from *Adktm1*$^{+/-}$ matings. 29 founder mice were obtained of which 3 founders carried the transgene in an *Adktm1*$^{+/-}$ background (*tgUbiAdk:Adktm1*$^{+/-}$) (Fig. 1*B*). These mice were then bred with *Adktm1*$^{+/-}$ mice with the aim of obtaining genetic rescue of the lethal ADK deficiency in *Adktm1*$^{-/-}$mice. Two of the three transgenic lines (#888 and #890) gave rise to all six possible genotypes (Fig. 1*B*) including *tgUbiAdk:Adktm1*$^{-/-}$. *TgUbiAdk:Adktm1*$^{-/-}$ mice (m#3, Fig. 1*C*) were identified in an *Adk* specific PCR analysis (Fedele et al., 2004) by a characteristic 840 bp band from the Adk knockout allele (m#2 in Fig. 1*C* as control), the lack of the 640 bp band indicative of the wild type allele (m#1 in Fig. 1*C* as control), and the presence of a 420 bp band (m#3 in Fig. 1*C*), from the *Adk* transgene. The genotypes were also verified in a transgene and knockout specific TaqMan^{TM} real time PCR analysis (data not shown).

TgUbiAdk:Adktm1$^{-/-}$ mice from both lines (888 and 890) had a normal life span, were fertile, and did not display any overt abnormalities. Thus, the introduction of the *tgUbiAdk* transgene provided full rescue of the lethal ADK deficiency phenotype described previously (Boison et al., 2002b).

ADK expression in *tgUbiAdk:Adktm1*$^{-/-}$ mice

To verify the ubiquitous expression of the *tgUbiAdk* transgene, Western Blot analysis was performed comparing ADK expression in heart, kidney, lung, brain, and liver from adult *tgUbiAdk:Adktm1*$^{-/-}$ (line 888) and wild type mice (Fig. 2*A*). In wild-

type mice ADK was highly expressed in the liver, while intermediate levels were seen in brain, lung, and kidney, and the weakest expression in heart. It is interesting to note that in brain only the small 38.7 kD isoform of ADK is expressed, while in the other organs both isoforms were found. As the short isoform of ADK was used for the transgenic rescue of ADK-deficiency, expression of ADK in the *Adk*$^{-/-}$ background (*tgUbiAdk:Adktm1*$^{-/-}$) gave rise to only one isoform of ADK (Fig. 2*A*, right panel). The Western Blot analysis revealed an increase in ADK expression in brain extracts from *tgUbiAdk:Adktm1*$^{-/-}$ mice (line 888), while liver levels of ADK were only partly reconstituted in these transgenic rescue mice compared to wild type (Fig. 2*A*). ADK levels in heart, kidney, and lung of the mutants appeared to be similar to those of wild-type mice (Fig. 2*A*).

Increased brain activity of ADK in *tgUbiAdk:Adktm1*$^{-/-}$ mice leads to increased levels of spontaneous locomotion

Spontaneous locomotor activity in wild-type (n = 7), *Adktm1*$^{+/-}$ (n = 9), and *tgUbiAdk:Adktm1*$^{-/-}$ mice (line 888, n = 6) was analyzed on three consecutive days. The three genotypes differed in the total amount of locomotion [$F(2,36) = 13.190$, $p < 0.001$] (Fig. 2*B*). *TgUbiAdk:Adktm1*$^{-/-}$ mice displayed heightened levels of spontaneous locomotor activity over a 24-h period in comparison to wild-type mice ($P < 0.001$, Scheffe test) while *Adktm1*$^{+/-}$ mice were indistinguishable from wild-type. The hyperlocomotion seen in *tgUbiAdk:Adktm1*$^{-/-}$ mice was stable over the three-day period of recording [Genotype x day, $F(4,36) = 1.033$, not significant (n.s.)]. In addition, the circadian rhythmicity of locomotor activity [Time, $F(23,414) = 24.512$, $p < 0.001$] was similar in the three genotypes [Gene x time, $F(46,414) = 0.653$, n.s.] (Fig 2*C*).

These data suggest that the general overexpression of ADK in *tgUbiAdk:Adktm1*$^{-/-}$ mice leads to hyperactive behavior, which may directly be due to lowered levels of the inhibitory neuromodulator adenosine.

Based on results from the Western Blot analysis and from the assessment of motor behavior, we further quantified brain ADK enzyme activity in those animals, used in the behavioral test. In addition, we added samples from line #890 of *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 3). Samples were analyzed by performing an enzyme-coupled bioluminescent assay for ADK in extracts from total adult brain (Fig. 2*D*). Accordingly, *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 6) displayed an increase in ADK activity of

110 % ($P < 0.001$ as compared to wild-type(n = 7)), while a non significant decrease of ADK activity (26 %) was seen in *Adk*$^{+/-}$ mice (n = 9) [$\chi^2(2) = 11.590$, $p < 0.003$, Mood Median test] (Fig. 2*D*). ADK activity from *tgUbiAdk:Adk*$^{-/-}$ mice of line 890 (n= 3) was elevated only by 6 % compared to control. Since *tgUbiAdk:Adk*$^{-/-}$ mice of line 888 displayed a more pronounced elevation of ADK activity compared to those of line 890 all further studies described here were performed with mice from line 888. Based on this increase of ADK activity in brain, *tgUbiAdk:Adk*$^{-/-}$ mice (line 888) constitute an important model to study adenosine-based regulatory processes in the brain.

Brains from *tgUbiAdk:Adktm1*$^{-/-}$ mice display ubiquitous expression of ADK

To analyze the brain-specific expression pattern of the *tgUbiAdk* transgene in an ADK-deficient background, ADK immunoreactivity was analyzed in coronal and sagittal sections taken from adult *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 3) and compared to those taken from wild-type mice (n =3). ADK immunoreactivity was visualized using a rabbit serum raised against recombinant ADK as described previously (Gouder et al., 2004).

Compared to the strong ADK immunofluorescence in evenly distributed astrocytes (Gouder et al., 2004) visualized in coronal sections from wild type mice (Fig. 3*A*), brains from *tgUbiAdk:Adktm1*$^{-/-}$ mice were characterized by a striking loss of ADK immunofluorescence in these astrocytes (Fig. 3*B*). This is due to the deficiency of the endogenous *Adk* gene. ADK immunoreactivity in *tgUbiAdk:Adktm1*$^{-/-}$ mice appeared as a diffuse and ubiquitous staining indicative of ubiquitous expression of the *tgUbiAdk*-transgene. In contrast to wild type mice (Fig. 3*A,C*), ADK immunoreactivity in the mutants also became apparent in neurons, which is particularly evident in pyramidal neurons of the CA3 and CA1 region of the hippocampal formation (Fig. 3*D*). Likewise, ADK immunoreactivity in astrocytes of the wild type cortex (Fig. 3*A*) was replaced by ubiquitous ADK immunoreactivity in the mutants, not restricted to specific layers of the cortex (Fig. 3*B*).

The ADK immunoreactivity data presented here clearly demonstrate a shift from the astrocytic endogenous ADK expression observed in wild type mice (Fig. 3*A,C*) to a ubiquitous transgenic overexpression of ADK in *tgUbiAdk:Adktm1*$^{-/-}$-mice (Fig. 3*B*) with particularly high levels of ADK-specific immunofluorescence in pyramidal neurons of the hippocampus (Fig. 3*D*).

Overexpression of ADK abolishes paired-pulse facilitation recorded in CA3 pyramidal cells

The increased ADK activity and increased locomotion in *tgUbiAdk:Adktm1*$^{-/-}$ mice (Fig. 2) suggest that these mice have lower brain levels of adenosine. Since ambient adenosine levels profoundly affect paired-pulse facilitation in mossy fiber–CA3 synapses (Moore et al., 2003) we compared paired-pulse ratios (PPR) in hippocampal cultures obtained from wild type and *tgUbiAdk:Adktm1*$^{-/-}$ mice. Whole-cell recordings of excitatory postsynaptic currents (EPSCs) in CA3 hippocampal neurons from wild type mice revealed facilitation of the second EPSC in response to paired stimulation (40 ms interval) of mossy fibers (PPR = 1.64 ± 0.18, n = 6) (Fig. 4*A,B*). In contrast, the paired-pulse ratio in slice cultures from *tgUbiAdk:Adktm1*$^{-/-}$ mice was shifted towards paired-pulse depression (PPD) (PPR = 0.85 ± 0.03, n =4) (Fig. 4*A,B*) and was significantly different from PPF in wild type ($p < 0.007$) (Fig. 4*B*). This finding showing a change in the basic properties of synaptic transmission in hippocampal neurons is consistent with an increase in brain adenosine concentrations mediated by the overexpression of ADK.

***TgUbiAdk:Adktm1*$^{-/-}$ mice do not display spontaneous seizure activity**

To investigate whether global overexpression of ADK in brain, particularly in the granular and pyramidal neurons of the hippocampus, leads to spontaneous seizure activity, we obtained hippocampal and cortical EEG recordings in *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 17). EEG patterns were comparable to those of wild-type control mice (n = 9) (Fig. 5). In a total of 153 hours of EEG recordings we were not able to detect a single seizure in the mutants. We conclude that neuronal overexpression of ADK does not cause spontaneous seizure activity.

KA-lesioned hippocampus in *tgUbiAdk:Adktm1*$^{-/-}$ mice does not display upregulation of astrocytic ADK

Intrahippocampal KA-injection in wild type mice eventually leads to astrogliosis and chronic recurrent seizure activity, which is paralleled by an overexpression of ADK in hippocampal astrocytes. To address the question whether overexpression of ADK is a cause or consequence of seizure activity we performed intrahippocampal injections of KA in *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 7) and wild-type mice (n = 4) as controls. Four weeks after KA-injection, brains of the mice were analyzed histologically. In Nissl

stained brain sections it became evident that *tgUbiAdk:Adktm1*$^{-/-}$ mice displayed a lesion of the KA-injected hippocampus (Fig. 6*A*), which was indiscernible from a wild-type lesion (Fig. 6*B*). Both lesions were characterized by a loss of neurons in the hilus and the CA1 region of the hippocampal formation. In addition, a dispersion of the granule cells and the pyramidal cells of the CA3 region became evident. These findings are in line with the development of hippocampal sclerosis, which is a hallmark of temporal lobe epilepsy. However, brain sections stained for ADK immunoreactivity, displayed significant differences between mutants and wild-type control. While wild-type mice were characterized by a profound increase of ADK in astrocytes of the KA-lesioned hippocampus (Fig. 6*D*), hippocampi of KA-injected *tgUbiAdk:Adktm1*$^{-/-}$ mice did not show any signs of astrocytic upregulation of ADK (Fig. 6*C*) a finding consistent with the genetic disruption of the endogenous astrocytic ADK in *tgUbiAdk:Adktm1*$^{-/-}$ mice. In addition, due to the loss of pyramidal cells in the CA1 and CA3 region of the hippocampal formation of KA-injected *tgUbiAdk:Adktm1*$^{-/-}$ mice, a loss of ADK immunoreactivity is observed (Fig. 6*A*), which is in marked contrast to the strong ADK immunoreactivity in neurons of untreated *tgUbiAdk:Adktm1*$^{-/-}$ mice (Fig. 3*D*). Therefore, despite the lack of the upregulation of endogenous ADK in *tgUbiAdk:Adktm1*$^{-/-}$ mice they still develop a typical KA-lesion comparable to that observed in wild-type controls.

Intrahippocampal KA induces astrogliosis in *tgUbiAdk:Adktm1*$^{-/-}$ mice

To investigate astrogliosis in KA-lesioned hippocampi of *tgUbiAdk:Adktm1*$^{-/-}$ mice in more detail we performed immunofluorescence studies with staining for GFAP, a marker for astrocytes. Analysis of hippocampal sections from naïve wild-type animals (n = 3) revealed a pattern of ordered astrocytes with more or less parallel processes on the stratum granulosum and individual star-shaped astrocytes in the hilus (Fig. 7*A*), while KA-injected wild-type animals (n = 3) 4 weeks after injection revealed massive astrogliosis in the dentate gyrus, observed as a dense network of astrocyte processes, which became particularly evident by a strong increase of immunofluorescence in the hilus (Fig. 7*B*). In contrast hippocampi of naïve *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 3) (Fig. 7*C*) displayed a slightly higher number of astrocytic processes, compared to control (Fig. 7*A*), while KA-lesioned *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 3) displayed a massive astrogliosis (Fig. 7*D*) exceeding the astrogliosis found in KA-lesioned control mice

(Fig. 7*B*). KA-induced astrogliosis in *tgUbiAdk:Adktm1*$^{-/-}$ mice was characterized by a dense network of astrocytic processes obscuring the boundary between hilus and stratum granulosum. In addition, hypertrophied giant astrocytes became apparent, which were not found to that extent in KA-lesioned wild-type animals. In conclusion, both naïve and KA-lesioned *tgUbiAdk:Adktm1*$^{-/-}$ mice displayed an increase in astrocytes compared to respective wild-type mice indicating that the absence of endogenous astrocytic ADK promotes astrogliosis.

KA-induced seizure activity in *tgUbiAdk:Adktm1*$^{-/-}$ mice

To investigate whether the lack of astrogliotic ADK expression and the overall over-expression of ADK in KA-lesioned hippocampi of *tgUbiAdk:Adktm1*$^{-/-}$ mice has an influence on seizure activity, we performed daily intrahippocampal EEG recordings during the first four weeks after KA-injection. All mice analyzed (n = 7) displayed seizure activity, which was comparable to that observed in KA-injected wild-type mice (n = 4) (Fig. 5, Table 1, Niky). To assess whether KA-induced seizures in *tgUbiAdk:Adktm1*$^{-/-}$ mice could be suppressed by inhibition of ADK, KA-treated *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 7) were injected with the ADK inhibitor 5-iodotubercidin (3.1 mg/kg, i.p.) 4 weeks after KA-injection when all mice experienced chronic seizure activity. Following the drug injection, seizures were completely suppressed in all animals (data not shown), a result which was similar to previously published data from seizure suppression by 5-iodotubercidin in KA-injected wild-type mice (Gouder et al., 2004).

Discussion

In the present investigation we describe the new mouse line *tgUbiAdk:Adktm1*$^{-/-}$, which constitutes a genetic rescue for the lethal deficiency of adenosine kinase (ADK) (Boison et al., 2002b). The rescue was achieved by introducing an *Adk* transgene (Fig. 1) which resulted in the ubiquitous expression of the short brain-specific isoform (38.7 kDa) of ADK (McNally et al., 1997) (Fig. 2*A*). In contrast, a liver-specific transgene failed to provide genetic rescue of the lethal ADK deficiency (unpublished results). Thus, the short isoform of ADK was sufficient and necessary to restore vital physiological functions in an ADK deficient background.

Neuronal overexpression of ADK in the brains of *tgUbiAdk:Adktm1*$^{-/-}$ mice

In addition to a profound overexpression of ADK in brains of *tgUbiAdk:Adktm1*$^{-/-}$ mice (Fig. 2*A*), detailed immunohistochemistry revealed a lack of endogenous astrocytic ADK (Fig. 3), which was replaced by the ubiquitously expressed transgenic ADK with particularly high levels in pyramidal neurons of the hippocampus (Fig. 3). In summary, ADK expression in brains of *tgUbiAdk:Adktm1*$^{-/-}$ mice is characterized by the following main features: *(i)* lack of endogenous ADK in astrocytes; *(ii)* expression of transgenic ADK in neurons; *(iii)* a more than twofold overexpression of ADK in whole brain (Fig. 2*D*). Thus, *tgUbiAdk:Adktm1*$^{-/-}$ mice provide for the first time the opportunity to study adenosine-based neuromodulation in the absence of endogenous astrocytic ADK expression.

Neuronal over-expression of ADK leads to hyperactivity

The changes of ADK expression in *tgUbiAdk:Adktm1*$^{-/-}$ mice, both in cell-type specificity as well as in quantity, are likely to affect the excitability of the brain via altered adenosine levels. In support of this notion several studies have shown that pharmacological manipulations resulting in increased extracellular adenosine result in enhanced presynaptic inhibition in the hippocampus and vice versa (Pak et al., 1994; Moore et al., 2003). Accordingly, in hippocampi from *tgUbiAdk:Adktm1*$^{-/-}$ mice, we found a lack of paired-pulse facilitation (Fig. 4), which indeed indicates a reduction of adenosine levels in this brain region. Consistent with our findings, adenosine A_1 receptor knockout mice lack adenosine-mediated inhibition of excitatory glutamatergic neurotransmission (Johansson et al., 2001). Since the overexpression of ADK in *tgUbiAdk:Adktm1*$^{-/-}$ mice is ubiquitous (Fig. 3*B*) and most likely affects all brain

regions, these mutants are expected to have decreased adenosinergic tone throughout the brain manifesting itself also in the observed increase in motor activity we report here. This finding is supported by several reports of adenosine receptor antagonism resulting in increased motor activity with much of the evidence pointing towards interactions with dopamine receptors (Popoli et al., 1998; Svenningsson et al., 1999; Ferre et al., 2001).

***TgUbiAdk:Adktm1*$^{-/-}$ mice: a new model to study epileptogenesis**

Since *tgUbiAdk:Adktm1*$^{-/-}$ mice lack endogenous astrocytic ADK and display prominent neuronal overexpression of ADK, these mutants represent an ideal model to study the cell-type specific roles of ADK in epileptogenesis. Brains of untreated *tgUbiAdk:Adktm1*$^{-/-}$ mice display a similar overexpression of ADK (2.2-fold compared to control, Fig. 2*D*) as hippocampi dissected from kainic acid (KA) injected epileptic wild-type mice (1.8-fold compared to control) (Gouder et al., 2004). However, in contrast to KA injected wild-type mice, which display chronic recurrent seizure activity (Bouilleret et al., 1999), *tgUbiAdk:Adktm1*$^{-/-}$ mice do not display spontaneous seizure activity (Fig. 5), although it is conceivable that decreased protective adenosine levels may result in a lower threshold for seizure induction (Etherington and Frenguelli, 2004). Thus, an overall overexpression of ADK, including neuronal overexpression, is not sufficient to cause spontaneous seizure activity. Rather, the cell type specific expression pattern of ADK and its endogenous gene regulation may play a crucial role in epileptogenesis. This conclusion is supported by other studies, which showed that the blockade of adenosine receptors as such is not sufficient to elicit seizures (Dunwiddie, 1999) and adenosine receptor antagonists do not seem to alter the course of a seizure. However, adenosine receptor antagonists can prolong epileptic seizures (Dragunow and Robertson, 1987) or convert recurrent seizure activity into status epilepticus (Young and Dragunow, 1994).

It is important to note that in contrast to endogenous ADK, which is upregulated in parallel to astrogliosis and seizure activity, the transgenic ADK appears to be constitutively expressed at high levels. Thus, 4 weeks after KA-injection into hippocampi of *tgUbiAdk:Adktm1*$^{-/-}$ mice no signs of upregulation of ADK were observed. Instead, a loss of ADK immunoreactivity was found in pyramidal neurons of the hippocampal formation, which is due to the KA-induced death of these ADK-

expressing cells, a finding which becomes evident by comparing Nissl- and ADK-stained sections from *tgUbiAdk:Adktm1*$^{-/-}$ mice (Fig. 6*B,D*).

Our previous findings in KA-injected wild-type mice demonstrated a parallel time course of ADK overexpression in hippocampal astrocytes, astrogliosis, and the progression of seizure activity (Gouder et al., 2004). Overexpression of ADK, by lowering protective endogenous adenosine levels, is likely to aggravate seizure activity and thus to contribute to epileptogenesis. However, based on these previous experiments (Gouder et al., 2004) it was not possible to determine whether overexpression of ADK in epileptic hippocampus was a cause or a consequence of astrogliosis and seizure activity. In our current study using KA injected *tgUbiAdk:Adktm1*$^{-/-}$ mice, we are able to demonstrate that overexpression of ADK in epileptic hippocampus is a consequence of astrogliosis and/or seizure activity. This conclusion is based on the following arguments: *(i)* after intra-hippocampal KA injection astrogliosis and seizures in *tgUbiAdk:Adktm1*$^{-/-}$ mice develop in the absence of endogenous astrocytic ADK and, therefore, in the absence of overexpressed ADK (Fig. 5-7); *(ii)* naïve *tgUbiAdk:Adktm1*$^{-/-}$ mice do not display spontaneous seizure activity (Fig. 5) despite the neuronal overexpression of ADK (Fig. 3*B*).

The reactive overexpression of ADK after KA-induced gliosis and seizure activity might be explained as a compensatory mechanism: The initial injection of KA into the hippocampus would cause immediate excitoxic neuronal death and status epilepticus triggering endogenous protective mechanisms including the release of adenosine (During and Spencer, 1992). Interestingly, a concomitant decrease in the expression of ADK is observed 1-24 hours after KA injection (Gouder et al., 2004), which would increase local levels of adenosine, presumably as a defense mechanism to arrest status epilepticus. As adenosine accumulates during status epilepticus and probably also during recurrent seizure activity, the brain may compensate by increasing the expression of ADK to remove excessive adenosine. Continued cell death, gliosis and reorganization of the hippocampus occur at the same time that ADK expression is increasing to abnormally high levels and, ultimately, chronic spontaneous seizures develop (Gouder et al., 2004). Since it is known that adenosine accumulates during seizure activity, it is conceivable that the chronic release of adenosine during the epileptic state continues to reinforce the overexpression of ADK. Thus, a vicious circle can be envisaged in that due to the overexpression of ADK the threshold for seizure

induction during interictal periods is reduced, thereby contributing to the progressive course of chronic epilepsy.

Efficacy of ADK inhibitors and adenosinergic drugs

As described above a dysregulation of the adenosinergic system was found to be associated with seizure activity (Rebola et al., 2003; Gouder et al., 2004). Despite our findings that overexpression of ADK in the brain may not be the cause of seizure activity, epilepsy-associated overexpression of ADK still provides a rationale for therapeutic intervention. The notion that dysregulation of the adenosinergic system also contributes to seizure activity and epileptogenesis is supported by the fact that drugs, which augment the adenosine response, suppress seizures. Thus, seizures can efficiently be suppressed by ADK inhibitors (Wiesner et al., 1999; Kowaluk and Jarvis, 2000; Gouder et al., 2004), by adenosine receptor agonists (von Lubitz et al., 1993; Monopoli et al., 1994; De Sarro et al., 1999; Huber et al., 2002; Gouder et al., 2003), and by intraventricular adenosine (Boison et al., 1999). Because of peripheral side effects of adenosine (Dunwiddie, 1999) effective strategies that enhance its protective effects in an area of adenosinergic dysfunction (i.e. a seizure focus) may require a novel approach, such as the local delivery of adenosine by cellular implants. This approach has proven successful in the rat kindling model of epilepsy (Huber et al., 2001). In addition, ADK inhibition in KA-treated *tgUbiAdk:Adktm1*$^{-/-}$ mice suppressed seizure activity, adding further evidence that adenosinergic therapy is beneficial.

In conclusion, *tgUbiAdk:Adktm1*$^{-/-}$ mice constitute a valuable new model to study epileptogenesis. In particular these mutants: *(i)* highlight the importance of ADK for the regulation of basic physiological functions; *(ii)* provide a model to study the effects of cell type specific expression of ADK in brain; *(iii)* provide evidence that overexpression of ADK in epileptic hippocampus is a consequence of astrogliosis and seizure activity; and *(iv)* facilitate the development of adenosine-based treatment for epilepsy, which may be of benefit to patients suffering from intractable epilepsy.

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Legends to Figures

Figure 1. Generation of *tgUbiAdk:Adktm1*$^{-/-}$ mice. *A,* The *tgUbiAdk* transgene (open boxes) was constructed by inserting the short isoform of a mouse *Adk*-cDNA between a human ubiquitin promoter (hUbi) and an SV40 polyA sequence (polyA). The transgene was flanked by loxP sites (grey triangles). *B,* Strategy to breed *tgUbiAdk:Adktm1*$^{-/-}$ mice. The *tgUbiAdk* transgene was injected into oocytes derived from *Adktm1*$^{+/-}$ mice. Transgenic founders, *tgUbiAdk:Adktm*$^{+/-}$ which were heterozygous for the transgene and which were heterozygous for the *Adk* knockout were backcrossed with *Adktm1*$^{+/-}$ mice. From these backcrosses offspring with six different genotypes were derived, among them *tgUbiAdk:Adktm1*$^{-/-}$ mice (m#3), which were viable like wild-type mice *Adk*$^{+/+}$ (m#1), in contrast to homozygous *Adk* knockout mice (*Adktm1*$^{-/-}$, m#2) which died shortly after birth. *C,* Representative PCR analysis of three selected animals (m#1-3). Top: Analysis of the *Adk* allele. The wild-type alleles (+/+) in m#1 are visualized as a characteristic 640 bp band, while the knockout alleles (-/-) in m#2 and m#3 are visualized as a characteristic 840 bp band. Bottom: Analysis of the *tgUbiAdk* transgene. The transgene specific PCR gave rise to a characteristic amplification product of 420 bp only in m#3, while the transgene was not detectable in m#1 and m#2. Thus, *tgUbiAdk:Adktm1*$^{-/-}$ mice constitute a viable genetic rescue of the lethal ADK deficiency (*Adktm1*$^{-/-}$). kb = 1 kb-ladder as size standard.

Figure 2. Ubiquitous expression of transgenic ADK results in hyperlocomotion. *A,* Western blot analysis of aqueous protein extracts derived from various organs from adult wild-type (wt)and *tgUbiAdk:Adktm1*$^{-/-}$ mice (tg888). Extracts corresponding to 15 μg of total protein each were probed with polyclonal rabbit antiserum raised against recombinant mouse ADK. Note the increase in ADK expression in the brain of *tgUbiAdk:Adktm1*$^{-/-}$ mice compared to wild type. *B,* The total locomotor activity (total number of interruptions of two consecutive light beams) over a 24 hour period was consistently enhanced in *tgUbiAdk:Adktm1*$^{-/-}$ (n = 6) ($p < 0.001$ as compared to wild-type mice (n = 7), Scheffe test) over 3 days while *Adktm1*$^{+/-}$ mice (n = 9) were indistinguishable from wild-type mice. *C,* The circadian rhythmicity of locomotor activity as assessed by mean relative locomotion per hour over a 12 hour dark/light cycle period (Zeitgeber time 0 is according to offset of light) was similar in the 3 genotypes ($F(46,414) = 0.653$, n.s.). *D,* Brain ADK enzyme activity as measured using an enzyme-coupled bioluminescent assay was increased in *tgUbiAdk:Adktm1*$^{-/-}$ mice (n = 6) while ADK enzyme activity was unchanged in *Adktm1*$^{+/-}$mice (n = 9) in comparison to wild-type mice (n = 7) ($p < 0.001$, Median test). Results are presented as relative light units (RLU's) normalized to lactate dehydrogenase (LDH) activity of the samples.

Results are expressed as means ± SE (Fig. 2B and C) and means ± SD (Fig. 2D).

Figure 3. Photomicrographs of ADK immunoreactivity in transverse brain sections of adult mice, processed for immunoperoxidase staining. *A,* Transverse section of a wild-type brain hemisphere showing a homogenous distribution of individual cells expressing ADK. *B,* Transverse section of a *tgUbiAdk:Adktm1*$^{-/-}$ brain hemisphere showing ubiquitous expression of the transgenic ADK coupled to a loss of punctate staining of endogenous ADK in individual cells. *C,* Wild-type hippocampus at higher magnification showing individual ADK-positive cells. *D,* Hippocampus from a *tgUbiAdk:Adktm1*$^{-/-}$ mouse showing the ubiquitous expression of transgenic ADK. Note the strong ADK immunoreactivity in pyramidal cell neurons. Scale bars: *A,B,* 800 μm; *C,D,* 400 μm.

Figure 4. Overexpression of ADK leads to a shift in the paired-pulse ratio measured in CA3 pyramidal cells. *A.* Representative traces of 50 averaged EPSCs in response to paired stimuli (40 ms interval) in wild type (n = 6) and *tgUbiAdk:Adktm1*$^{-/-}$ (n = 4) mice. *B.* Pooled data showing a shift from paired-pulse facilitation in wild type to paired-pulse depression in *tgUbiAdk:Adktm1*$^{-/-}$ mice.

Figure 5. Representative cortical and hippocampal EEG recordings taken from wild-type and *tgUbiAdk:Adktm1*$^{-/-}$ mice. *A.* Base line brain activity in untreated wild-type mice from hippocampal (H, upper traces) and cortical (C, lower traces) EEG recordings. *B.* Base line brain activity in untreated *tgUbiAdk:Adktm1*$^{-/-}$ mice. Note the similarity of basal brain activity compared to wild-type. *C.* Typical seizure in an epileptic wild-type mouse 4 weeks after KA-injection. *D.* Typical seizure in an epileptic *tgUbiAdk:Adktm1*$^{-/-}$ mouse 4 weeks after KA-injection.

Figure 6. Changes in hippocampal cyto-architecture and ADK immunoreactivity induced by intrahippocampal kainic acid injections. *A,B.* Cresyl-violet staining of transverse sections through brains of *tgUbiAdk:Adktm1*$^{-/-}$ mice (*A*) and wild-type mice (*B*) three weeks following an intrahippocampal KA-injection. Note that the progression of the lesion does not differ in the 2 genotypes. *C,D.* ADK immunoreactivity of transverse sections through brains of *tgUbiAdk:Adktm1*$^{-/-}$ mice (*C*) and wild-type mice (*D*) three weeks following an intrahippocampal KA-injection.

Scale bar: 500 µm

Figure 7. GFAP immunofluorescence, as seen by confocal laser-scanning microscopy. Transverse brain sections of KA-injected wild-type and *tgUbiAdk:Adktm1*$^{-/-}$ mice taken 4 weeks after the injection and those from naïve control animals of both genotypes were stained for the astrocyte marker GFAP. Optical sections were digitized at high magnification. *A,* Dentate gyrus of a naïve wild-type animal. *B,* Dentate gyrus of a KA-injected animal. Note the massive gliosis characterized by the swelling of cell bodies and the enlargement of astrocytic processes. *C,* Dentate gyrus of a naïve *tgUbiAdk:Adktm1*$^{-/-}$ mouse. Note the higher number of astrocytic processes, compared to *A. D,* Dentate gyrus of a KA-injected *tgUbiAdk:Adktm1*$^{-/-}$ mouse. Note the massive enlargement of astrocytic cell bodies. The boundary between hilus and stratum granulosum is no longer visible. sg, stratum granulosum; hi, hilus; Scale bar: 50 µm.

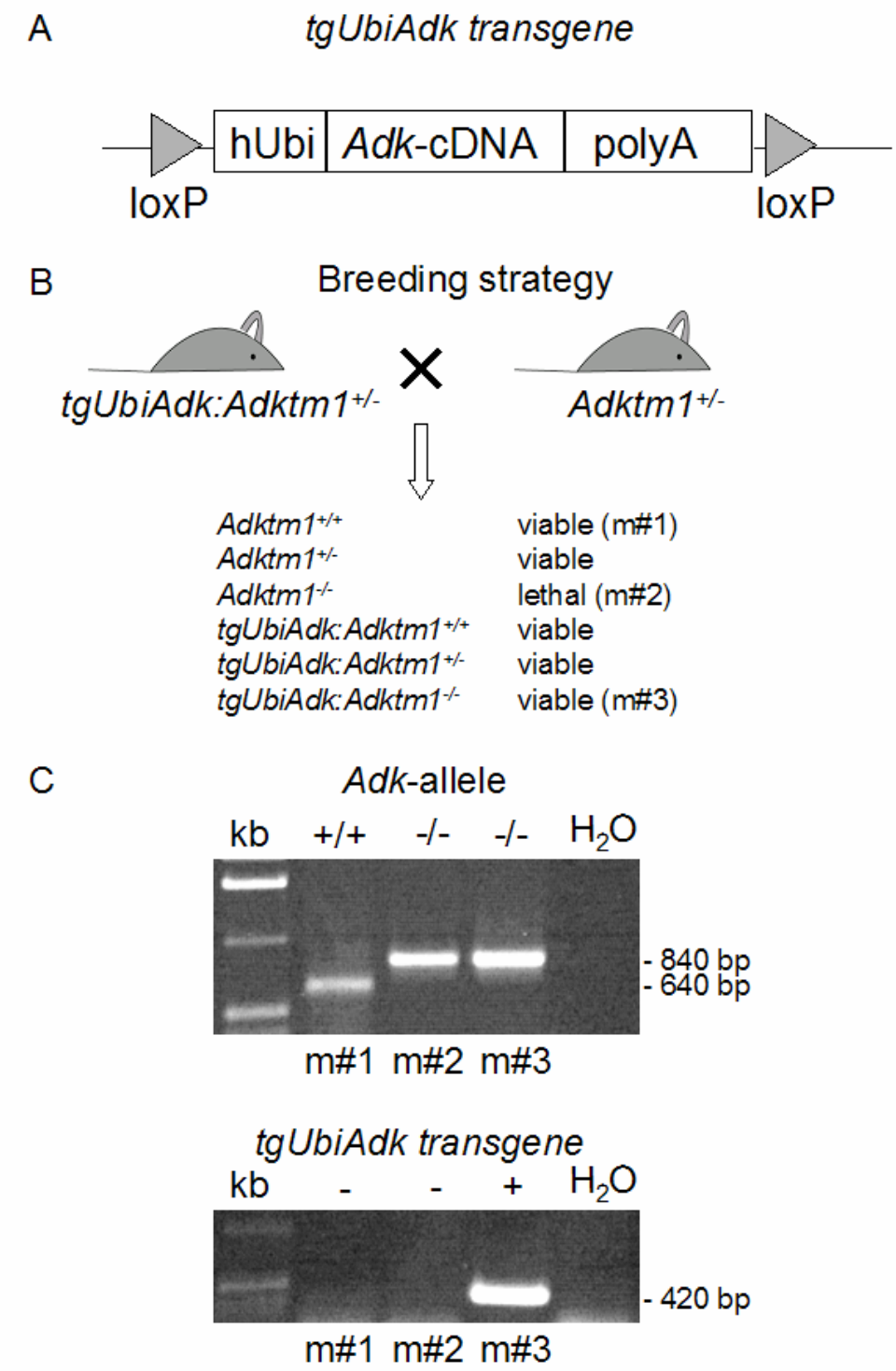
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Fig. 1

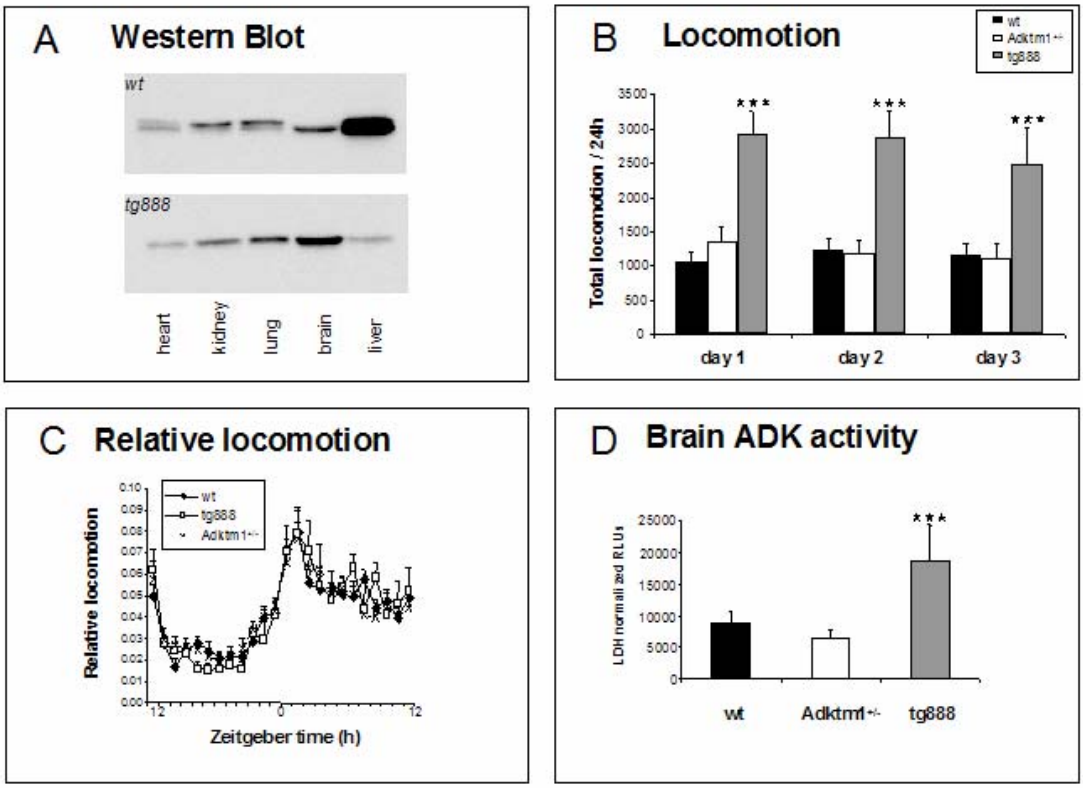
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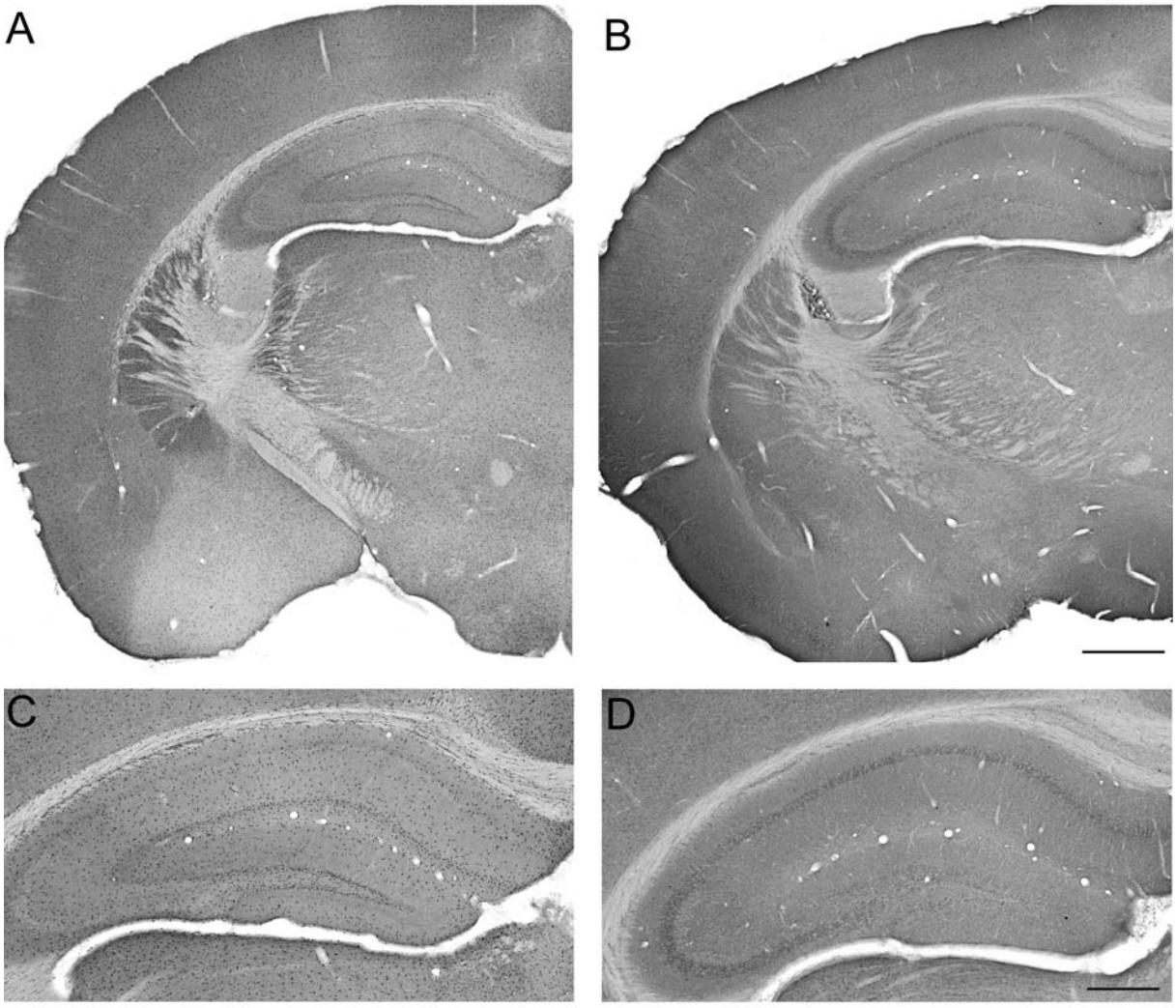
Fig. 2



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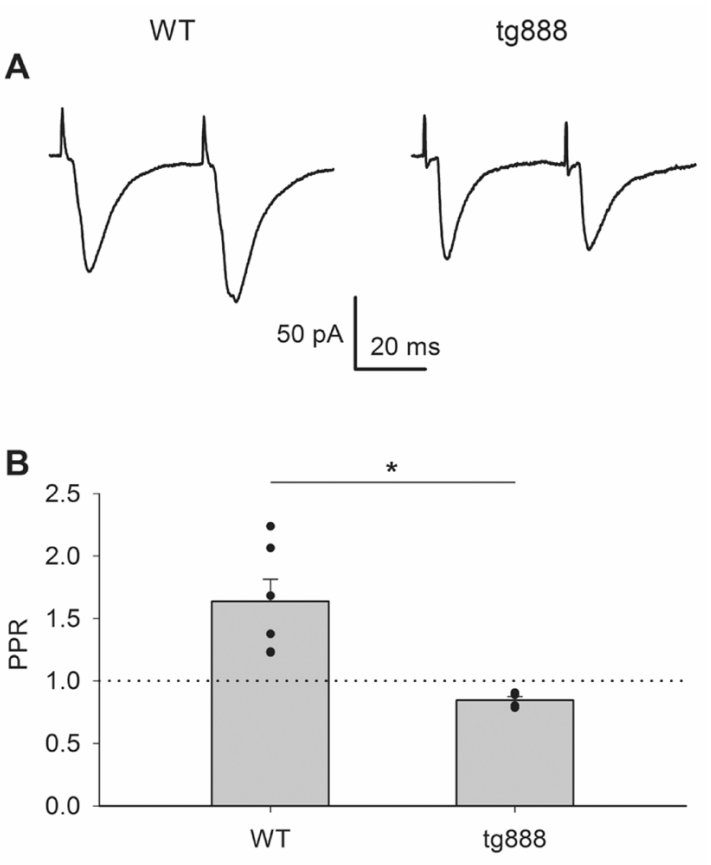
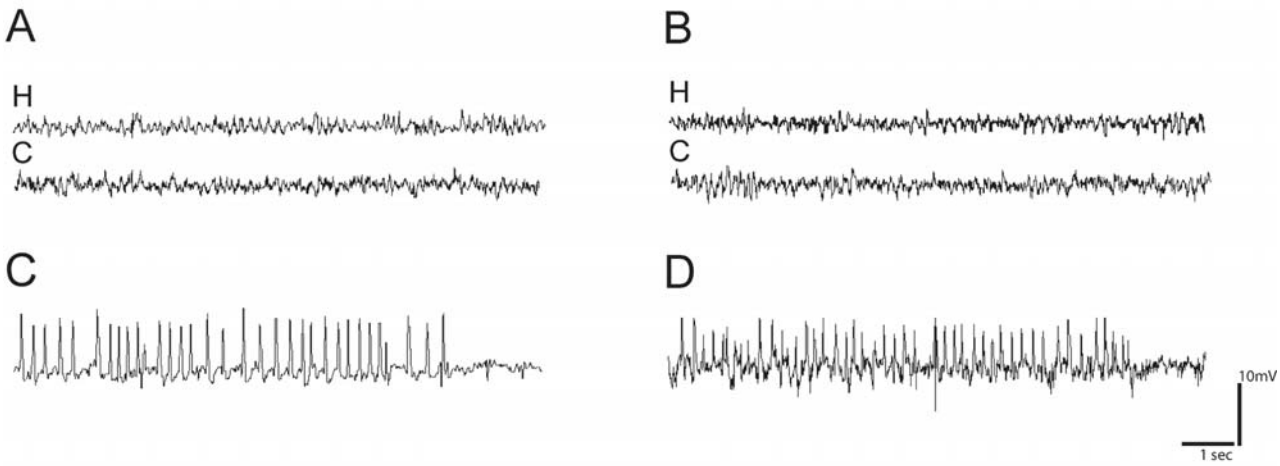


Fig. 4



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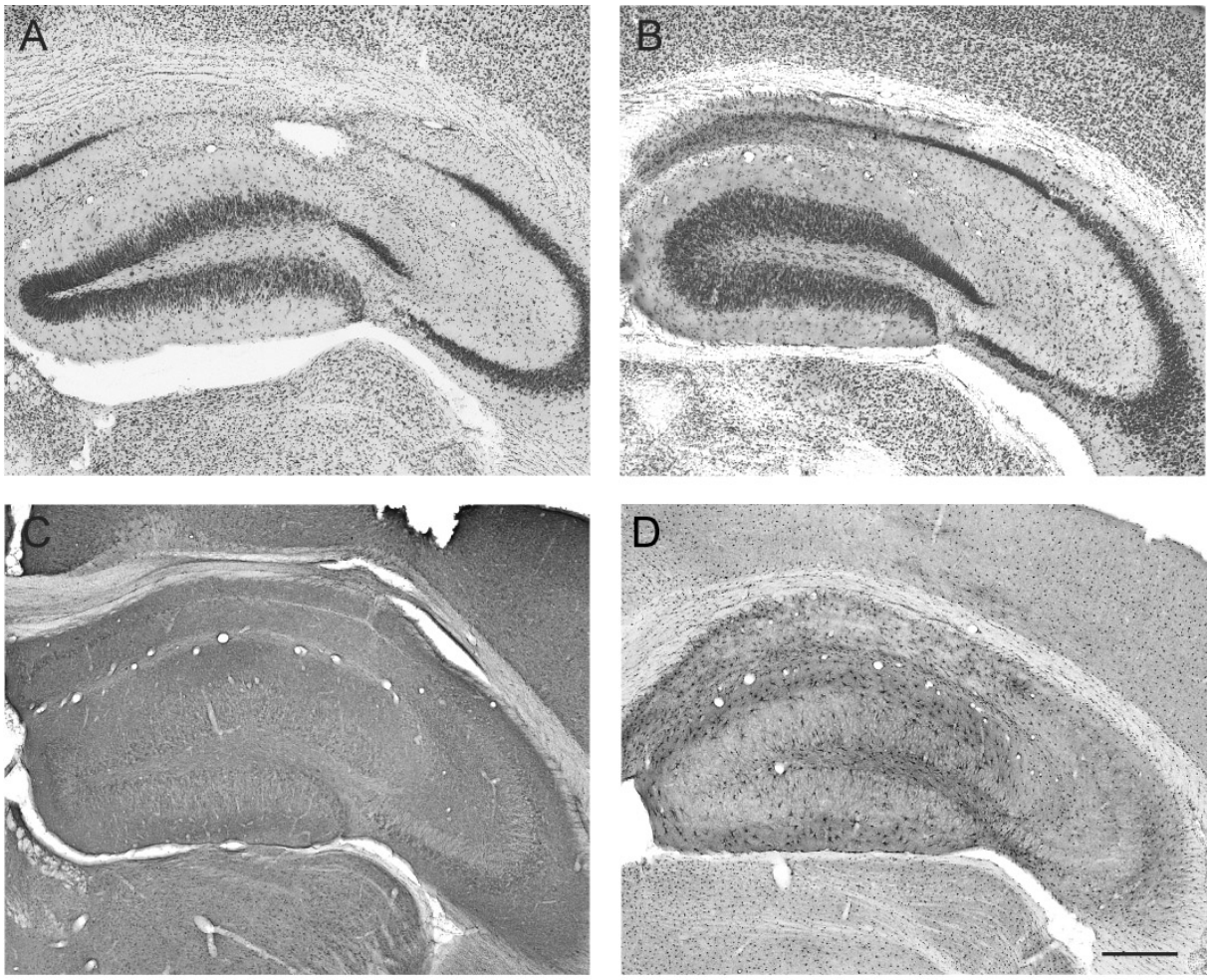

Fig. 6

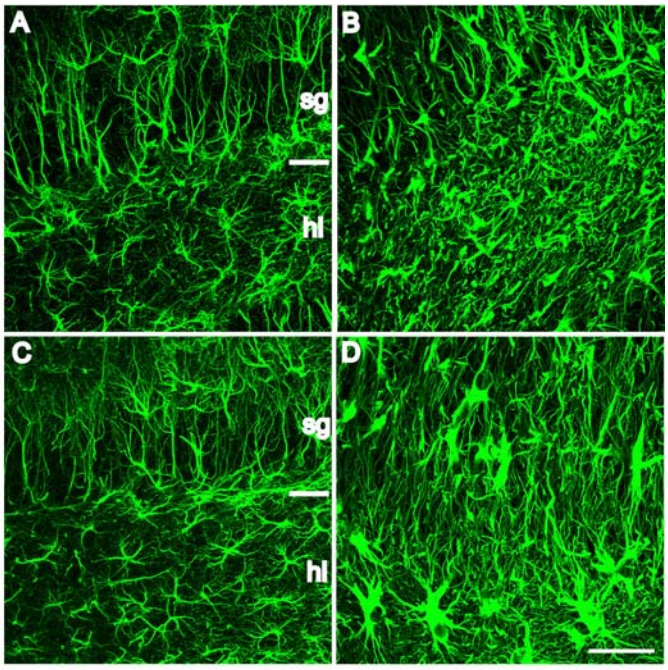

Fig. 7

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Research Publications

1. Bobkov IuV, **Grishin AA**, Rogachevskaia OA, Kolesnikov SS Proton-activated K+-channels of frog taste receptor cells Biofizika. 1999 Sep-Oct; 44(5), pp 870-879.
2. **Grishin AA**, Gee CE, Gerber U, Benquet P. Differential calcium-dependent modulation of NMDA currents in CA1 and CA3 hippocampal pyramidal cells. J Neurosci. 2004 24(2), pp 350-355

3. **Grishin AA**, Benquet P, Gerber U Muscarinic receptor stimulation reduces NMDA responses in CA3 hippocampal pyramidal cells via Ca^{2+}-dependent activation of tyrosine phosphatase (submitted in Neuropharmacology)

Abstracts

1. A.A. Grishin, Yu. V. Bobkov, S.S. Kolesnikov
   Feasible role of proton-activated K^+ channels of frog taste receptor cells in acid stimuli detection// in Abstracts of IV Pushchino conference of young scientists (in Russian) Puschino, April 19-23, 1999
2. Yu.V. Kim, Yu.V. Bobkov, A.A. Grishin, S.S. Kolesnikov P2Y receptor-mediated calcium release and ion channel modulation in mouse taste receptor cells. //(abstract) in 13th international symposium olfaction & taste ISOT 2000 and 14th European chemoreception research organization congress ECRO 2000, 20th to 24th July 2000, the Brighton Centre, Brighton, UK. pp. 158-159
3. Yu.V. Kim, Yu.V. Bobkov, A.A. Grishin, S.S. Kolesnikov Coupling P2Y receptors to the activation of a Cl current in mouse taste receptor cells// (abstract) in international symposium Intracellular Signaling in Plant and Animal Systems, Minsk (Belarus), September 24-26, 2000 p. 15
4. A.A. Grishin, P. Benquet, M. Mori, U. Gerber Differential Ca^{2+}-dependent muscarinic regulation of NMDA receptors in CA1 and CA3 pyramidal cells//(abstract) in 33rd annual meeting of Society for Neuroscience, November 8-12, 2003, New Orleans (USA).
5. A.A. Grishin, P. Benquet, U. Gerber Muscarinic receptor stimulation reduces NMDA responses in CA3 hippocampal pyramidal cells via Ca^{2+}-dependent activation of tyrosine phosphatase//(abstract) in 34th annual meeting of Society for Neuroscience, October 22-27, 2003, San Diego (USA).

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